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Poorthuis, R. B. (2013). *Nicotinic acetylcholine receptor modulation of attention behavior and prefrontal cortical circuits*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam]. Vrije Universiteit.

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Nicotinic acetylcholine receptor modulation of attention behavior and prefrontal cortical circuits

Rogier B. Poorthuis

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The research described in this thesis was carried out at the department of Integrative Neurophysiology embedded in the Centre for Neurogenomics and Cognitive Research at the Neuroscience Campus Amsterdam

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About the cover:
The cover displays an artistic impression of a mouse performing an attention task. The mouse is running through a network of neurons located in the prefrontal cortex that might be important for encoding its own behavior.

VRIJE UNIVERSITEIT

Nicotinic acetylcholine receptor modulation of attention behavior and prefrontal cortical circuits

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. F.A. van der Duyn Schouten,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Aard- en Levenswetenschappen
op donderdag 12 september 2013 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Rogier Bernard Poorthuis

geboren te Hilversum

promotor: prof.dr. H.D. Mansvelder

“The truth outlasts every scientist”

“It is through science that we proof,
but through intuition that we discover”
Henri Poincaré

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General introduction

Poorthuis, R.B.

Chapter 1

Elements of this text have been published in:
Biochemical Pharmacology 2009 Oct 1;78(7):668-676
Poorthuis RB*, Goriounova NA*, Couey JJ*, Mansvelder HD

1. Rationale: Why pay attention?

In everyday life our brain is continuously overwhelmed by sensory inputs; sounds, images, smells, tastes, touches and information about ourselves. Although the brain is incontestably an enigmatic organ and its efficiency in processing sensory information is remarkable, it has a limited capacity to process many features simultaneously. It compensates for this limited bandwidth of our thoughts by an exceptional cognitive function: attention (Buschman and Miller, 2010). Attention can be defined as the ability to filter out irrelevant information in order to structure our behavior in time along an internal goal (Miller and Cohen, 2001). As such, two processes are relevant here that control attention. Firstly, cognitive factors such as knowledge, expectations and goals determine which features in the outside world you will favor over others (top-down attention). Secondly, sensory information that is entering from the environment and marked by our brain as relevant (bottom-up attention) (Corbetta and Shulman, 2002). The interplay between these processes determines what we focus on. It makes it possible to concentrate on prolonged tasks, like reading a book, but at the same time deal with novelty and unexpectedness, i.e. the smell of fire while reading the same book. The latter influences attention strongly. It enables us to rapidly interact with a dynamic environment (Corbetta and Shulman, 2002; Fuster, 2001; Miller and Cohen, 2001), but also be easily distracted by modern technology like our mobile phone.

A great deal of knowledge about the mechanisms of these functions comes from variations in the ability of humans to pay attention. Natural factors like genetic constitution play a role in determining variation in attention behavior in humans and rodents (Bidwell et al., 2007; Loos et al., 2012). At the lower end of this distribution of attention performance, people might encounter disability in everyday life, i.e. people suffer from brain disease. Disorders in the domain of attention and executive control are among the most disabling in cognitive dysfunctioning and include Alzheimer's, ADHD and schizophrenia (Barkley, 1997; Greene et al., 1995; Hutton et al., 1998). While the underlying pathophysiology of these diseases is beyond the scope of this thesis, it is relevant to point out that in all of these diseases altered functioning of the cholinergic system has been found. In Alzheimer's patients, neurons that release acetylcholine, the endogenous signal molecule acting on nicotinic and muscarinic acetylcholine receptors, degenerate and lead to a loss of ability to sustain attention (Perry and Hodges, 1999; Whitehouse et al., 1981). In addition, the number of nicotinic receptors has been reported to decline in patients (Guan et al., 2000; Whitehouse et al., 1986). Alzheimer's patients are therefore treated with drugs that enhance cholinergic signaling and nicotinic receptor functioning (Levin and Rezvani, 2002). Schizophrenic patients show reduced nicotinic receptor expression (Guan et al., 1999; Leonard et al., 2000) and the locus of the gene for a particular subtype of the nicotinic receptor is associated with genetic transmission of this disease (Freedman et al., 2001). In addition, both ADHD and schizophrenic patients show a high incidence

of smoking (Leonard et al., 2000; Milberger et al., 1997). This 'self-treatment' regime indicates the involvement of the cholinergic system in these diseases. Therefore, many nicotinic-like drugs are developed in order to treat these people some of which have proven successful (Levin and Rezvani, 2002; Wilens and Decker, 2007). In contrast, in healthy populations, nicotine seems to have a detrimental effect on attention, as people who smoked during adolescence show more often attention related problems in later life (Jacobsen et al., 2005; Newhouse et al., 2004b). In conclusion, the cholinergic system plays a pivotal role in cognitive performance and focusing attention.

While many studies on attention and the function of the cholinergic system exist in humans, there are limitations to the questions one can answer in this approach. These include the execution of behavioral experiments in a standardized laboratory environment and the use of isogenetic models, the study of molecular and physiological properties of underlying neuronal networks and the inability to manipulate the genome of humans. Elucidating these detailed mechanisms is necessary to understand how specific proteins, and the nicotinic receptor in particular, function in the brain. Ultimately this information leads to a better understanding of the underlying mechanisms of attention in humans and possibly the design of more specific pharmacological agents targeting the cholinergic system. In this thesis, studies are performed concerning the underlying mechanisms of nicotinic receptor function in the modulation of attention behavior and prefrontal cortical circuits in mice.

As these questions build on prior knowledge, the introduction serves to give an overview of the role of prefrontal cortical networks in attention behavior in rodents. I will outline how the function of the prefrontal cortex is determined by its functional architecture and describe why acetylcholine takes a central role in performing attention tasks and modulating the underlying circuits. In addition, I will point out why the cholinergic system in the brain is so sensitive to the influence of nicotine and how this changes attention behavior and cortical circuits on the short- and long-term.

2. The prefrontal cortex plays a central role in attention

The cortex is the most recently evolved structure of the brain and is especially well developed in mammals and humans in particular. Along with the expansion of our brain during ancient times came an increase in complexity and performance of behavior. With the evolvement of visual, auditory and olfactory processing systems animals became able to explore larger areas (Douglas and Martin, 2012), perhaps like internet networks enable us to reach the whole world in modern days. This also required a more complex internal representation system, which enabled cognitive functions such as (working) memory, motivation, planning and attention. The prefrontal cortex is generally seen as the hallmark of evolution making many of these complex cogniive functions possible. As highlighted above, a reduction in prefrontal cortical (PFC) function is detrimental for functioning in society and is a core feature

in brain diseases like ADHD, Alzheimer's and schizophrenia. Evidence from people with damage in prefrontal structures strengthens our idea about the role of the prefrontal cortex in cognitive control and attention. While Phineas Gage might be the most famous person suffering from frontal lobe damage (his brain was pierced by an iron rod which led to altered behavior like personality changes, impulsivity and attention problems (Macmillan, 2000), also in scientific laboratory environments altered cognitive function after dorsolateral prefrontal damage has been investigated (Bechara et al., 1994). Most characteristic is the inability to attain future goals (Bechara et al., 1994; Miller, 2000). Subjects are more impulsive (choose immediate reward over higher rewards on the longterm) caused by an insensitivity to future consequences (Bechara et al., 2000) and have an inability to focus on a task when irrelevant features compete for their attention (Duncan et al., 1996; Miller, 2000).

The dorsolateral primate cortex is analogue to the medial prefrontal cortex in rodents. It comprises three stacked brain structures along the dorsoventral axis; the anterior cingulate, prelimbic and infralimbic cortex (Figure 1) (Groenewegen and Uylings, 2000). Similarly as in humans its involvement in attention has been substantially proven (Muir et al., 1996b; Robbins, 2002). In rodents the most applied paradigm to test attention is the 5-choice serial reaction time task (5-CSRTT) (Robbins, 2002), analogue to the continuous performance task in humans (Beck et al., 1956). In this visuospatial attention task a rodent is trained to respond to a brief light stimulus (usually ~1 second). Its attention is directed toward five holes in which this cue can be presented. Consequently, when it responds in the hole where the light was presented it obtains a reward. The paradigm yields several measures of attentional performance (Figure 2). First, accuracy of responding is determined by the amount of times the rodent responded in the illuminated hole over the total number of correct and incorrect responses. Second, errors of omission count the absence of responding to any stimulus hole when the cue is presented. Errors of omission reflect a lack to sustain attention across the whole task, which usually consists of 60-100 trials. It can be indicated as a gross impairment in attention when other factors influencing this measure are excluded. Disruptions in locomotor behavior and motivational behavior influence this trait as well, but can be controlled for by comparing reaction time to the cue-light and magazine latency, respectively, or by performing complementary behavioral tasks taxing these functions (Robbins, 2002). Last, the task assesses the ability of the rodent to restrain from responding before the cue-light is presented (usually 5 seconds after the trial starts). When the animal makes an impulsive response it does not receive a reward and the task is paused. The 5-choice is often used to assess this unbeneficial form of decision making (Eagle and Baunez, 2010).

Many studies tried to identify the locus of attention in this paradigm. Most substantial effects on attention have been found by lesions of the frontal cortices (Robbins, 2002). Lesions in all prefrontal cortical structures lead to a decrement in choice accuracy and perseverative responding (Muir et al., 1996b). A more

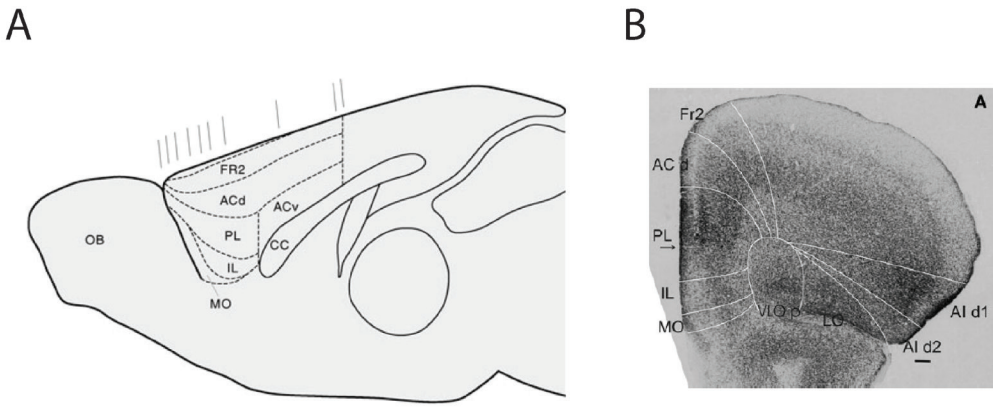

Figure 1. Prefrontal cortical areas in the mouse brain.
(A) Sagittal view of medial prefrontal cortical areas in the mouse.
(B) Coronal slice of the medial prefrontal cortical areas in the mouse defined by cytoarchitectonic borders (taken from van de Werd et al. 2010). OB=Olfactory bulb, FR2=Frontal Area 2, ACd=Dorsal Anterior Cingulate Cortex, ACv=Ventral Anterior Cingulate Cortex, PL=Prelimbic Area, IL=Infralimbic Area, CC=Corpus Callosum, MO=Medial Orbital Frontal Cortex, LO=Lateral Orbital Frontal Cortex.

restricted function was found for the anterior cingulated cortex in premature responding, indicating that this region is in particular important for regulating cognitive control (Muir et al., 1996b). Next to a reduction in accuracy, an additional increase in omissions after medial(m)PFC lesions has been reported (Broersen and Uylings, 1999; Muir et al., 1996b)(Broersen and Uylings, 1999; Muir et al., 1996b). The latency for a correct response was not altered in these studies, indicating spared motor functions (Broersen and Uylings, 1999; Muir et al., 1996b). These studies show a causal relationship between intact prefrontal networks and attention behavior. However, a central question that has not been revealed yet is how neurons generate activity supporting attentional performance and how the anatomical organization of this interaction is brought about. Overlapping impairments in attentional modalities across the different regions suggest a tight cooperativity between prefrontal cortical structures underlying attention behavior, which has been subject of recent studies.

Two studies elucidated the role of the prefrontal cortical network in supporting preparatory attention (top-down attention), stimulus detection and error-related learning (Totah et al., 2012; Totah et al., 2009). In the seconds before cue presentation activity changed in the mPFC and anterior cingulated cortex neurons when an actual correct hit was made. The change in activity depended on attentional performance as it was absent in omitted trials. These findings indicate that the PFC displays preparatory activity which supports proper attentional performance (Totah et al., 2009). Interestingly, as many units showed increased firing as well as decreased firing, indicating that inhibition in the PFC might play an important role in regulating preparatory activity (Totah et al., 2009). Activity was also changed after making an

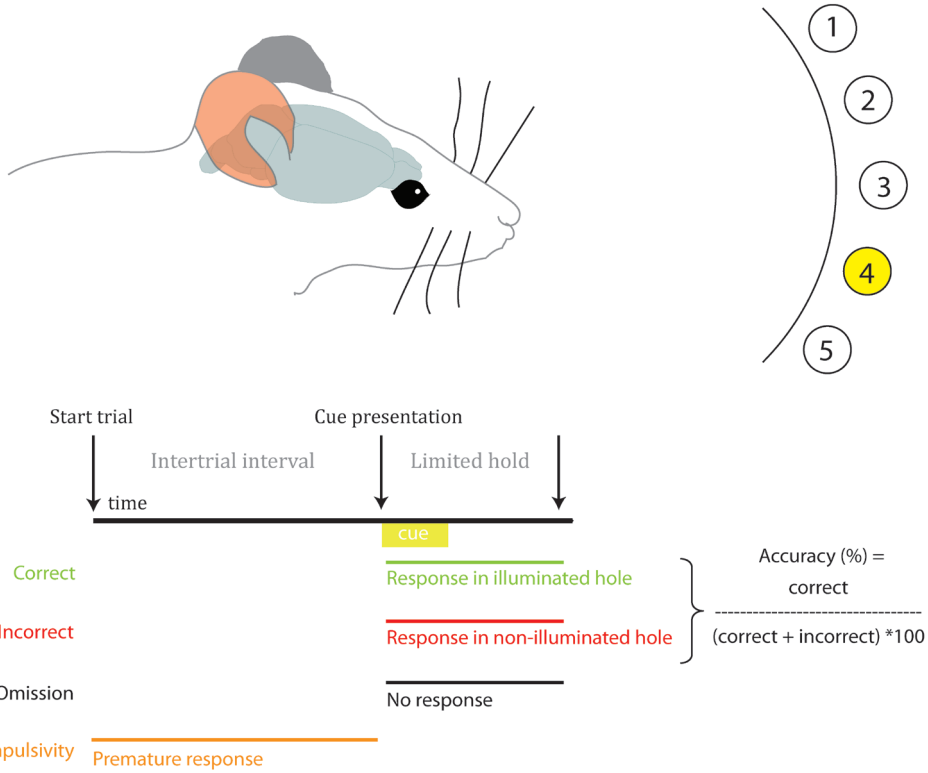

Figure 2. Overview 5-CSRTT and attentional parameters.
Schematic depiction of the 5-CSRTT procedure in which attention is directed towards five holes that each can be illuminated (see text; for review see [8]). Different attentional parameters can be scored in the paradigm. Accuracy is the percentage of correct responses divided by the sum of correct and incorrect responses, i.e. a response in a non-illuminated hole. Omissions count the total amount of trials on which no response is made in one of the nose-poke holes upon stimulus presentation. An impulsive response is made when a rodent makes a response before presentation of the visual target.

actual nose poke, and in the cingulated cortex in particular also when an error is made, suggesting that the PFC is involved in stimulus detection, and selecting and learning of appropriate action. Computations in the PFC are complex as it has been shown that the mPFC and cingulated cortex highly interact during preparatory activity. The magnitude of synchrony between these networks can predict behavioral outcome (Totah et al., 2012). All together, these data suggest a role for the prefrontal cortex in preparatory attention, stimulus detection and error-related learning. How the prefrontal cortex performs this dazzling computing task remains elusive, but how relevant output is generated from the input received by the PFC highly depends on functional connectivity of underlying neuronal networks. As explained below, these networks show a stereotypical buildup.

3. Functional architecture of neuronal circuits in the prefrontal cortex

The cortex is horizontally organized into a sheet of six layers and also connect vertically to facilitate communication between modules or columns (Mountcastle, 1997). Incoming information is differentially computed within these layers (de Kock et al., 2007). The prefrontal cortex is a superhub and shows reciprocal connectivity with many brain areas. Unlike primary sensory areas, the prefrontal cortex is a multimodal association cortex and its main function lies in integrating sensory, limbic and visceral information to alter behavioral state of an animal and select appropriate motor programs (Groenewegen and Uylings, 2000). The cortex is mainly composed of pyramidal neurons (~80%), dynamically connected to local interneurons (~20%). Determining the connectivity and modulation these neurons is key in elucidating the computational nature of the cortex. Pyramidal neurons are excitatory and transmit information across layers, to subcortical areas and other cortical areas by releasing glutamate. Pyramidal neurons are at first sight a more homogenous group than interneurons. However, pyramidal neurons differ in their cellular properties depending on their target structure (Hattox and Nelson, 2007). In addition, pyramidal neurons connecting to different areas are also differentially modulated by neurotransmitters (Dembrow et al., 2010). Pyramidal neurons in the prefrontal cortex show laminar specificity according to their projection target (Gabbott et al., 2005). Superficial neurons mainly project to the amygdala. Middle layered neuron project to hypothalamus and basal ganglia and deep layer neurons to the medial dorsal thalamus. The prefrontal cortex in turn is updated with information coming from many different brain areas like the (medial dorsal) thalamus and hippocampus (Little and Carter, 2012; Rotaru et al., 2005). Relevant output is send after transforming these input signals. Attention will highly depend on the interaction between the input and output structures. Supporting this many of these structures, like the amygdala, striatum and medial dorsal have been implemented in regulating attentional performance (Robbins et al., 2002). As an example, after amygdala lesions attention performance is impaired (Holland et al., 2000) and pharmacological manipulations of the striatum also lead to altered attention performance (Baunez and Robbins, 1999).

Critical in the computational function of the cortex are interneurons. A highly diverse group of neurons that (in the cortex) mainly project locally to alter pyramidal neuron function (Markram et al., 2004). Interneurons are mostly inhibitory and hence serve to restrict excitation of neurons and can do this in a compartmentalized way (Kawaguchi and Kubota, 1997). In this way excesses like overstimulation or complete absence of neuronal encoding is prevented. Different hardwired configurations exist depending on the functional connectivity. If a pyramidal and interneuron are interconnected, pyramidal neuron firing can lead to feedback inhibition onto the principal neuron. Afferent inputs into the cortex are mostly balanced by feedforward inhibition. In this configuration axons target both a pyramidal neuron and an

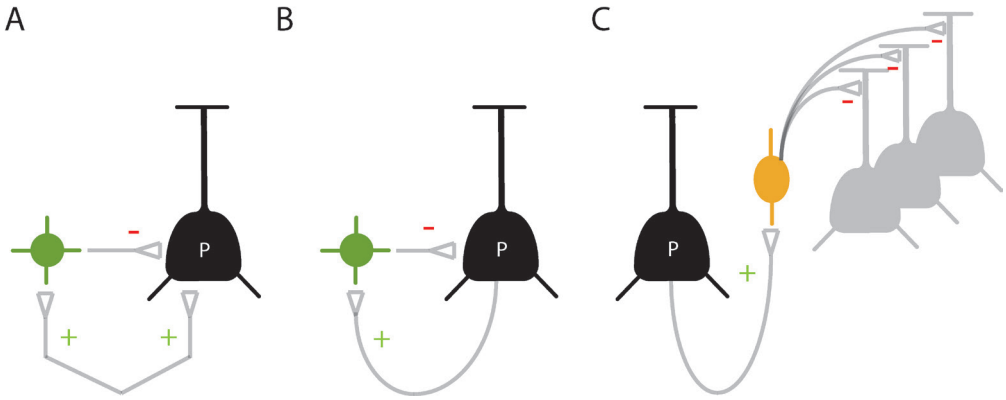

Figure 3. Schematic wiring diagrams of cortical circuits.
(A) Afferent inputs into cortical circuits are organized in a feedforward configuration. Excitatory signals onto pyramidal neurons are balanced by quick feedback inibition. The same afferent axon excites an interneuron connected to the same pyramidal neuron it targets.
(B) Interconnected pyramidal and interneurons lead to feedback inhibition ensuring that activity of a pyramidal neuron is followed by inhibition.
(C) In the lateral inhibition configuration activity of the pyramidal neurons inhibits neighbouring pyramidal neurons. This could lead to selection of only a few relevant outputs.

interneuron connected to the pyramidal neuron. Excitation is thereby followed by inhibition to increase temporal fidelity of spike timing (Adesnik et al., 2012; Porter et al., 2001; Pouille and Scanziani, 2001; Sun et al., 2006). Finally, activity in pyramidal neurons can serve to activate interneurons that inhibit neighbouring pyramidal neurons. Lateral inhibition could function to ensure the selection of only one (or a few) relevant outputs (Figure 3) (Adesnik et al., 2012).

4. Cholinergic modulation of the prefrontal cortex and attention behavior

The dependence of attention behavior on intact prefrontal cortex networks and associated changes in neuronal activity poses the question how proper performance-dependent activity is generated in the PFC. Neuromodulators are pivotal in altering function of neuronal networks depended on the behavioral state of an animal. Particularly interesting in this respect is the finding that the removal of cholinergic innervation to the prefrontal cortex leads to a strong decrease in firing of PFC neurons related to performance in the attention task (Gill et al., 2000). It argues for a central role of acetylcholine in modulation of attention behavior that many studies underpin.

The prefrontal cortex receives dense cholinergic innervation from a set of nuclei located in the basal forebrain (Mesulam et al., 1983; Saper, 1984). Cholinergic fibers are found abundantly in layers V en VI and at a more moderate density in layers I-III, opting for a layer specific modulation of the network (Saper, 1984). Functionally, a loss of basal forebrain neurons by lesions interferes with performance in tasks

designed to assess various aspects of attentional performance (Muir et al., 1994; Muir et al., 1993; Muir et al., 1992; Robbins et al., 1989). More refined experiments showed the selective involvement of cholinergic projections to the cortex (Chiba et al., 1999), especially in sustained attention (McGaughy et al., 1996; McGaughy and Sarter, 1998) and the ability to divide attention among stimuli from different modalities (Turchi and Sarter, 1997). Impairments of cholinergic innervation of the prefrontal cortex mainly affect attention performance, as impairments in for example working memory are absent or only mild and transient (Hasselmo and Sarter, 2010). Animals with impaired cholinergic innervation were also tested on a sustained attention task which requires reporting both signal and non-signal trials. Interestingly, cholinergic depletion significantly impaired the detection of cues on signal trials but animals were not impaired in reporting the absence of a cue. This shows that decreased cholinergic signaling particularly effects the detection of external cues and does not affect 'blank' trials, which do not require cue-detection. This indicates that the cholinergic system is in particular involved in cue detection (Hasselmo and Sarter, 2010; McGaughy et al., 1996). It has long remained enigmatic how this relates to acetylcholine release in the brain.

Early microdialysis studies clearly showed ACh efflux in the PFC during attention tasks (Arnold et al., 2002; Himmelheber et al., 2001). Increases in acetylcholine are positively related to increases of the demand on attention (Kozak et al., 2006; Kozak et al., 2007; Passetti et al., 2000). Due to these observations the dominating view was that acetylcholine signaling fluctuates on a scale of minutes, thereby bringing the cortex in an 'aroused' state (Figure 4) (Sarter et al., 2009). The recent development of a electrochemical sensor measuring acetylcholine release in real time drastically changed this view (Parikh et al., 2007). It revealed that rats that attend cues showed sharp rises (or transients) in acetylcholine concentrations in the prefrontal cortex (Figure 4), which were absent when a cue was missed (Parikh et al., 2007). In addition, these transients were not observed in motor areas, indicating the regional specificity of this type of modulation. Hence, acetylcholine release during cue-detection indicates that it encodes defined cognitive operations. Rises in this neurotransmitter correlated with a shift in behavior, indicating that they encode incorporation of a cue into new-goal directed behavior (Parikh et al., 2007; Sarter et al., 2009). These findings led to the hypothesis that acetylcholine fluctuates on different timescales. Phasic cholinergic signaling is important to encode defined cognitive operations to incorporate the detection of a cue into new goal-directed behavior, whereas ambient levels could encode the 'readiness' to process relevant sensory inputs.

Eliciting cholinergic transients in the PFC relies on the activation of glutamate receptors. Lesion studies point towards a circuit in which cholinergic transients are elicited by stimulation of afferents from the medial dorsal thalamus (Parikh et al., 2010; Parikh et al., 2008). These terminals have been shown to contain nAChRs themselves (Couey et al., 2007; Gioanni et al., 1999; Lambe et al., 2003) and when

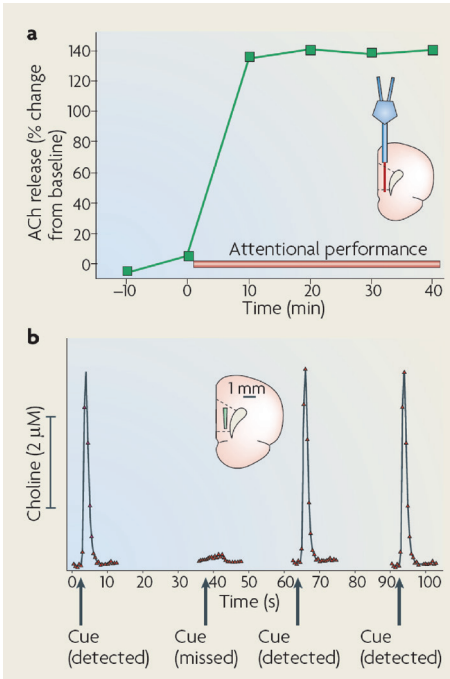

Figure 4. Dynamics of acetylcholine release.
(A) Typical illustration of acetylcholine release during attention tasks as measured with in vivo microdialysis. The low temporal resolution of this technique led to the idea that acetylcholine levels change slowly during attention tasks and mediate 'aroused' states.
(B) Recent experiments using choline sensitive microelectrodes revealed that cholinergic signaling happens on the scale of seconds and that transient increase in acetylcholine in particular mediate cue-detection. Ideas emerge that acetylcholine release is not merely brining the cortex in an aroused state through volume transmission, but encode defined cognitive operations on a short timescale.

activated elicit cholinergic transients in the PFC (Parikh et al., 2010). Cholinergic transients might be enhanced by selective stimulation of nicotinic receptors and this has been hypothesized to underlie increases in cue detection or attention performance (Howe et al., 2010; Parikh et al., 2010). It points to a situation in which acetylcholine is important to regulate bottom-up attention by encoding sensory stimuli entering the PFC that need to be incorporated to cause a shift in behavior (Hasselmo and Sarter, 2010). In addition, changing ambient levels of acetylcholine might act on these thalamocortical circuits to alter 'readiness' of the PFC to process relevant sensory information (Hasselmo and Sarter, 2010; Parikh and Sarter, 2008). In this way top down attention interacts with bottom-up attention to regulate overall attentional performance. The circuitry involved in regulating ambient levels of acetylcholine in the PFC is not known, but might depend on the reciprocal connectivity between the basal forebrain and the PFC.

In conclusion, altered PFC activity is associated with attention performance and cholinergic signaling plays an important role in altering network activity. Which receptors receive cholinergic signals and how they translate this into functional neuronal activity remains unanswered. Acetylcholine stimulates both metabotropic muscarinic and ionotropic nicotinic receptors (Picciotto et al., 2012). The fast kinetics of cholinergic release upon cue-detection and modulation of attention behavior by the psychoactive substance nicotine (Hahn et al., 2003b) suggests that ionotropic nicotinic receptors play a major role. In chapter 2 we aim at answering this question by assessing attention behavior of mice deficient for specific subtypes of the nicotinic receptor. It also probes the question to what extent cholinergic signals are restricted in space and time and how neuronal circuits are organized to regulate this. The following section highlights several aspects regarding this matter.

5. Modes of cholinergic release: synaptic and volume transmission

Despite a wealth of data on the role of cholinergic signaling in attention coming from microdialysis, amperometry, lesioning and pharmacological studies, still very little is understood about the temporal and structural specificity of the cholinergic system. Neurotransmitter systems convey much information through conventional synapses, i.e. an axon terminal (presynaptic element) is opposed to functional receptors (postsynaptic element). While this way of transmitting signals permits a very tightly regulated form of information processing, it does not allow for groups of neurons to be brought into a different functional state. The conventional view about the functioning of classical neurotransmitters (GABA and glutamate) has always been through point-to-point communication (Okubo and Iino, 2011), but this view has now been challenged (Okubo et al., 2010; Olah et al., 2009), showing that signals are conveyed extrasynaptically and to groups of neurons. In contrast, neuromodulators have always been thought to act through volume transmission, bringing networks in a different state in a nonspecific manner. The main reasons that determined this view were deduced from the idea that acetylcholine signals were slow and heightened for minutes during attentional task. In addition, the cholinergic system appears highly unspecific in terms of its connectivity to the cortex (Sarter et al., 2009). The basal forebrain diffusely projects to the whole cortex (Woolf, 1991). Although an innervations map exists along the dorsoventral, lateralmedial and rostrocaudal axis and basal forebrain neurons preferentially innervate one part of the cortex, still neighbouring neurons can innervate very different regions (Sarter et al., 2009; Zaborszky, 2002). This might point to a non-specific communication system, but it is probably far from that. Histochemical quantification of cholinergic varicosities in the cortex revealed synaptic structures opposing release sites, although contradiction arises concerning the number that has been reported. There is evidence for a low structural correlation with synaptic structures (~17%), indicatory of diffuse release mechanisms (Mechawar et al., 2000; Mechawar et al., 2002), as well as a high incidence of synaptic structures (60-70%) opposing acetylcholine releasing terminals (Smiley et al., 1997; Turrini et al., 2001).

Recent new tools offer exciting insights into these questions. With optogenetics one can specifically activate cholinergic axons in the cortex (Kalmbach et al., 2012) and study release properties from its original source. ACh release elicits dual component nicotinic receptor currents in interneurons in the cortex (Arroyo et al., 2012). Currents with a fast rise time were conducted by α7 nAChRs and slow components by β2* nAChRs. β2* nAChRs carried more charge and led to more pronounced firing (Arroyo et al., 2012). The receptors showed different spatiotemporal properties. When acetylcholine-esterase inhibitors were applied signals through β2* nAChRs were prolonged whereas α7 nAChRs remained unchanged. This might indicate that

β2* nAChRs are located at a greater distance from the release site of ACh (Bennett et al., 2012). In addition, to mimick neuronal α7 nAChR kinetics in nucleated patches a high concentration of ACh was needed (~200 μM). Concentrations found in the extracellular fluid are generally lower and therefore suggest that synaptic release might stimulate α7 nAChRs. These answers remain inconclusive though, since similar experiments have not been performed for β2* nAChRs and α7 nAChRs might respond less to increased levels of ACh due to their fast desensitisation kinetics (Bennett et al., 2012).

Interesting would be to know how restricted the modulation of the PFC circuitry by acetylcholine is. Sustained levels of acetylcholine are present in the extracellular space, albeit with low concentrations of acetylcholine (Parikh et al., 2007; Sarter et al., 2009), but this does not reveal how high ACh concentrations are near release sites and where they originated. Volume transmission and phasic release might encode different processes. Rapid rises in acetylcholine on seconds timescales encode defined cognitive operations (cue detection) and slower changes in ACh might encode the readiness of processing new sensory information predictive of cue detection (Parikh et al., 2007; Sarter et al., 2009) and motivation state (Paolone et al., 2012). This might indicate that high affinity receptors are more likely to be activated during changes in ambient levels of ACh, while high and low-affinity nicotinic receptors are activated during fast changes in ACh. The enzyme acetylcholinesterase, that degrades acetylcholine, might be permissive for local actions on these receptors. This is the fastest enzyme in the brain and is highly present in the prefrontal cortex. Also the neural circuitry of regulating PFC levels of acetylcholine seem to be more specific as previously thought, as nucleus accumbens activity specifically modulates PFC ACh levels, but not other cortical circuits (Alexander et al., 2009). Many questions remain unanswered about the spatiotemperal organization of the system, but will rely on determining the connectivity patterns that regulate basal forebrain afferent activity in the PFC.

In conclusion, data thus far do not support a definite conclusion but mixed synaptic and non-synaptic release mechanisms might contribute to the modulatory effects of ACh. In chapter four we add to this question an experiment in which we investigate the activation properties of different receptor subtypes of the PFC by different modes of ACh release (fast versus slow) and show that they are highly different for receptor subtypes. Nicotinic receptors are molecularly and physiologically divers and consequently have very different roles in network modulation.

6. Structure, function and diversity of nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) belong to the cys-loop ligand-gated ion-channel family (Gotti et al., 2009). This group of pentameric transmembrane proteins form a water-filled pore upon binding of neurotransmitter after which charged ions can flow over the membrane. Twelve genes have been identified encoding neuronal

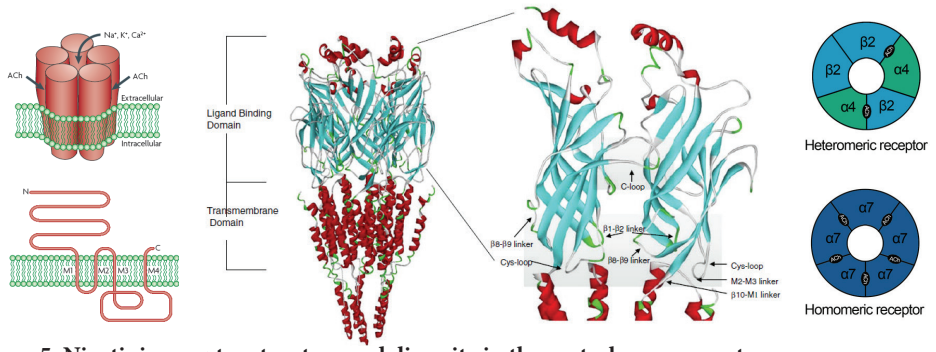

Figure 5. Nicotinic receptor structure and diversity in the central nervous system.
(A) Nicotinic receptors are pentameric channels and upon binding of acetylcholine open to form a waterpore non-specifically conducting cations. They are made of five subunits of which one is shown. A subunit contains four transmembrane domains which are for example important for determining ion-selectivity.
(B) Crystal structure of a nicotinic receptor
(C) Stoichiomertry of the two most abundant nicotinc receptors. The pentameric protein can be homomeric, made of five identical alpha7 aubunits with five binding sites. Heteromeric receptors are made of alpha and beta subunits and contain two agonist binding sites.

nicotinic receptors [for review see (Le Novere et al., 2002)]. Each gene encodes a subunit of the receptor that can be classified into α-type subunits and non-α-type subunits, based on the presence or absence, respectively, of a pair of cysteine amino acids (Chavez-Noriega et al., 1997; Gotti et al., 2009; Le Novere et al., 2002), reviewed in (Changeux and Edelstein, 2001; Gotti and Clementi, 2004; Gotti et al., 2006; Hogg et al., 2003). This cysteine pair is important for agonist binding and it has been thought therefore that α-subunits at least in part regulate this process. In the central nervous system 9 α-subunits (α2–α10 encoded by CHRNA2–10) and 3 β-type subunits (β2–β4; CHRNB2–4) are expressed. These subunits assemble in different stoichiometries to form the pentameric channel, and the subunit composition of nAChRs varies depending on the brain region [for review see (Alkondon and Albuquerque, 2004; Gotti et al., 2009; Grady et al., 2002; Le Novere et al., 2002; McGehee, 2002; Mineur and Picciotto, 2008; Wonnacott et al., 2005). Nicotinic receptors can either assemble as homomeric or heteromeric channels. Heteromeric channels are formed by a combination between α and β subunits, whereas some α subunits can assemble into a homomeric channel. When opened, the nicotinic receptor is a cation selective channel which permits flow of Na^+, K^+ and Ca^{2+} across the membrane. At normal resting membrane potential this leads to a depolarizing current.

The impact of nAChR activation on neuronal function strongly depends on the subunit composition of the nAChRs. Each subunit combination has its own activation and desensitization characteristics and has different single channel conductance and agonist selectivity, potentially leading to different kinetics of depolarizing currents in the target cell (McGehee and Role, 1995a; Millar and Gotti, 2009). The two most abundant nicotinic receptors in the brain are receptors that contain α4β2 or α7 subunits

(Figure 5). α4β2* receptors show a prolonged current opening upon activation by ACh, have a high affinity for nicotine and desensitize at low concentrations of nicotine, corresponding to blood concentrations experienced by smokers (Chavez-Noriega et al., 1997; Millar and Gotti, 2009). In contrast, the homomeric channels containing α7 subunits show rapid activation and desensitization kinetics when stimulated by ACh, have a lower affinity for nicotine and do not desensitize at low nicotinic concentrations (Mansvelder and McGehee, 2002b; Wooltorton et al., 2003). These phenomena will have significant impact on how these receptors are activated in neuronal networks (see below). Another distinctive feature of these receptors is their permeability for calcium. Amino acids lining the pore of the protein largely determine the ion-selectivity of the channel. α7 nicotinic receptors are highly permeable for calcium compared to α4β2* receptors (Fucile, 2004). They serve, therefore, a distinguished role because this calcium influx can influence cellular processes like neurotransmitter release and synaptic plasticity directly. It has been suggested that α7 nicotinic receptors perform a complementary role to NMDA receptors. These channels are also calcium permeable, but are only opened at a more depolarized membrane potential (Dingledine et al., 1999). Hence, despite their low carriage of charge across the membrane (Bennett et al., 2012), which might lead to a minor impact on excitablity, they might serve an important local modulatory role in regulating synaptic plasticity (Ji et al., 2001).

In the cortex nicotinic receptors are highly expressed across all cortical regions (Millar and Gotti, 2009), and in the PFC in particular. Nicotinic receptor expression is found across all cortical layers of the PFC (Gioanni et al., 1999). Next to α4β2 and α7 subunits, in the cortex the accessory α5 subunit is also highly expressed (Millar and Gotti, 2009), and this has also been shown for the PFC (Counotte et al., 2012a). α5 subunits are preferentially expressed in deep cortical layers (Wada et al., 1990; Winzer-Serhan and Leslie, 2005). Indeed it has been shown that pyramidal neurons in layer VI contain this subunit (Bailey et al., 2010; Kassam et al., 2008). This accessory subunit is assembled together with α4β2 subunits and that gives this nicotinic receptor unusual properties. It shows a heightened sensitivity for ACh and protects nicotinic receptors from desensitization (Bailey et al., 2010; Grady et al., 2012). α5 subunit expression has been shown to be much lower in other cortical layers (Wada et al., 1990; Winzer-Serhan and Leslie, 2005). How these different subunits contribute to attention behavior and regulate the underlying neuronal circuitry is the main subject of this thesis. In addition, we investigate in chapter 5 the sensitivity of these receptors for nicotine and how this changes the responsiveness of the circuitry to cholinergic signaling.

7. Evidence for the involvement of nicotinic receptors in attention behavior

There is ample evidence that nAChR activation in general affects attentiona; performance (Levin et al., 2006; Mansvelder et al., 2006; Mirza and Stolerman, 1998,

2000), but much less is known about the nAChR subtypes and brain areas involved. As described, several studies point to a specific role of cholinergic signaling in the medial prefrontal cortex and attentional performance (Hahn et al., 2003b; McGaughy et al., 2002; Parikh et al., 2010b). However, only a limited number of studies have addressed the role of nAChR subtypes in the PFC and their role in attention behavior. Infusion of α-bungarotoxin, an α7 nicotinic receptor antagonist, into the prefrontal cortex impairs performance in a delayed response task, which requires effortful processing for response selection (Granon et al., 1995). β2-containing nAChRs have also been implicated in mediating effects of nicotine on attentional performance. Nicotine decreased response latency and reduced incorrect responses in the 5-choice serial reaction time task. These effects of nicotine were completely antagonized by dihydro-β-erythroidine (DHβE), a specific blocker for β2-containing nicotinic receptors. In this study, methyllycaconitine (MLA), a rather selective blocker of α7-containing nAChRs, did not alter the effects of nicotine (Blondel et al., 2000). However, genetic approaches assessing the role of nAChRs have shown that α7 receptors do have a role in attention. Knockout mice lacking the gene for the α7 nAChR subunits showed impaired task acquisition and a higher rate of omissions in the 5-choice serial reaction time task (Hoyle et al., 2006; Young et al., 2007). In addition, genetic deletion of the α5 subunit leads to a decrement in response accuracy (Bailey et al., 2010). Since the mice used for these studies lacked α7 and α5 nAChR expression throughout their brains, it is not known whether the impairment in attention performance was attributable to the lack of α7 receptors specifically in the PFC, let alone whether the effects were attributable to a specific type of cells in the PFC neuronal circuits. If nicotinic compounds are to be designed as cognitive enhancers for therapeutic use, a detailed understanding of how nAChR activation affects attention and PFC microcircuits will be indispensible. In particular mechanisms need to be pinpointed towards a specific locus in the brain. The experiments described in chapter 2 aim at answering this question by investigating attentional performance in the 5-choice reaction time task of β2, α7 and α5 null mice. Single gene expression using lentiviral vectors was used to investigate region specific effects of these receptors. To understand how nicotinic receptors can regulate information processing in the cortex we need to zoom in to the microcircuit level.

8. Nicotinic receptors and their modes of action

The large body of evidence demonstrating that nAChRs can affect cognitive processes offers an enticing chance to link protein function to complex behavior (Levin et al., 2006; Mansvelder et al., 2006; Maskos, 2007; Picciotto, 2003). However, to understand the mechanisms involved at the level of neuronal networks, there are several bridges yet to be built. Typically, a cortical microcircuit consists of a set of excitatory and inhibitory neurons that are interconnected using highly dynamic

connections. To understand how nAChR activation in the prefrontal cortex affects cognitive behavior, an understanding is needed of how prefrontal cortical microcircuits generate output from the inputs they receive. One of the challenges is to distinguish the cell types and the connectivity patterns that are present in the prefrontal cortex circuitry. The next step is to understand how nicotinic receptors alter the functionality of these neuronal circuits. General principles on how nicotinic receptors affects microcircuits in the brain will depend on (i) which cell types in the circuits express nicotinic receptors and (ii) from which subunits these receptors are made of. The latter strongly determines activation and desensitization kinetics, agonist sensitivity and ion-specific channel conductance. (iii) The sub-cellular location of the receptor will determine what stage of information processing is affected, since nicotinic receptors can be found on dendritic, somatic, axonal and presynaptic compartments. Nicotinic AChRs expressed in axons or axon terminals can alter release of neurotransmitter at specific sites, even independent of action potential depolarization (Kawai et al., 2007; Lambe et al., 2003; Lena et al., 1993; Mansvelder and McGehee, 2000; McGehee et al., 1995). This often results in an increased probability of release at these sites, changing the way information carried by these synapses enters the cnortex and is processed. Alternatively, nicotinic AChRs can alter whole neuron functions by changing resting membrane potentials. These nicotinic currents can drastically alter the availability of Na^+ and K^+ channels for action potential generation (through inactivation), affect resting membrane potential (depolarization), and even potentially alter regional voltage signals via shunting (Couey et al., 2007; Dani and De Biasi, 2001; Mansvelder and McGehee, 2002; Pidoplichko et al., 1997). (iv) Given the fact that nAChR can be continuously activated by endogenous ACh, nAChR desensitization can affect ongoing neuronal activity just as nAChR activation (Mansvelder et al., 2002; Mansvelder and McGehee, 2002; Picciotto et al., 2008; Wooltorton et al., 2003). Therefore, the dynamics of endogenous cholinergic signaling will play an important role in the effects of exogenously administered nicotinic compounds. How the interplay between nAChR activation and deactivation on a subsecond time scale by endogenous ACh and exogenously applied agonists will affect cortical neuronal network activity remains to be elucidated. (v) Finally, it will be of significance whether nAChRs are activated by direct synaptic contact or via a slower process like volume transmission. Spill over and diffusion of ACh will result in different activation and desensitization profiles compared to targeted fast synaptic release, as indicated previously.

These factors will combine to alter neuronal network properties and will be central to understanding nicotinic receptor modulation of and nicotine its effects on higher cognitive functions. Although we are only beginning to understand how nicotine is affecting neuronal circuits in the prefrontal cortex, several features have now been uncovered, some of which show similarities to cholinergic modulation of other cortical areas, emphasizing that common principles may exist guiding nAChR modulation of cortical circuits and these will be discussed in the next sections.

9. Nicotinic receptor modulation of thalamocortical communication

One of the first recognized functions for nAChRs in the central nervous system was its role in enhancing neurotransmitter release (McGehee et al., 1995). As first described in chicken medial habenula-interpeduncular synapses and later in the mossy fiber synapse in the rat hippocampus, nicotine augments synaptic release of glutamate via presynaptic receptors (Gray et al., 1996; McGehee et al., 1995). The facilitating effect of nicotine was dependent on the extracellular calcium concentration, and nicotine application leads to a higher calcium signal in mossy fiber boutons. Depending on the subunit composition and precise location, nicotinic receptors can enhance presynaptic neurotransmitter release either through depolarization or direct calcium influx or both (Dajas-Bailador and Wonnacott, 2004). Nicotinic AChRs on axonal projections play a key role in regulating the transmission of thalamic information to the cortex (Clarke, 2004; Gioanni et al., 1999; Kawai et al., 2007; Lambe et al., 2003; Metherate, 2004). Transdermal administration of nicotine to non-smokers does not affect cochlear activity but does affect the neural transmission of acoustic information (Harkrider et al., 2001). A similar result was observed in the rat auditory cortex (Liang et al., 2008). Critically, in this study antagonists of nAChRs reduced the evoked signal in the cortex, suggesting that endogenous ACh acts through nAChRs to regulate thalamic transmission. The barrel cortex of the rat is perhaps the best studied model of thalamocortical transmission, and here to nicotinic agonists can alter cortical processing. Topical application of nicotinic agonist to the exposed cortex *in vivo* increased the size of a whisker's functional representation in the cortex (Penschuck et al., 2002). Earlier recordings in thalamocortical slices from the barrel cortex support this result by demonstrating that thalamic synapses, unlike intracortical synapses, are modulated by nAChRs (Gil et al., 1997). Even in the visual cortex, nicotine can increase responsiveness to visual stimuli (Disney et al., 2007; Lawrence et al., 2002).

Although the prefrontal cortex is thought to be a higher order processing area, it receives thalamic input from the dorsal medial nucleus of the thalamus. Thalamocortical glutamatergic transmission to the prefrontal cortex is augmented by the activation of nAChRs (Couey et al., 2007; Gioanni et al., 1999; Kassam et al., 2008; Lambe et al., 2003; Vidal and Changeux, 1993). Autoradiographic labelling of nAChRs was reduced after lesions in the medial dorsal thalamus (MDT). This suggested that nAChRs are present on thalamocortical terminals and could potentially alter thalamic information processing in the PFC. When neurons in the MDT are stimulated in vivo action potentials are elicited in the prefrontal cortex. Infusing nicotine locally into the PFC enhanced the response elicited in the prefrontal cortex. Microdialysis experiments showed that nicotine induced glutamate release in the PFC which could be blocked by DHβE (Gioanni et al., 1999). Lesioning the MDT strongly reduced the augmentation of glutamatergic inputs to layer V pyramidal neurons by nicotine

(Lambe et al., 2003). This suggests that among the glutamatergic inputs received by layer V pyramidal neurons, nicotine selectively stimulates thalamic inputs. As with thalamocortical inputs to somatosensory cortex, the nAChRs responsible for the augmentation by nicotine were located away from the presynaptic terminal, most likely on the axons themselves (Kawai et al., 2007; Lambe et al., 2003). These nicotinic mechanisms differ from the mechanisms by which nicotine increases excitatory transmission in the hippocampus and VTA, where activation of α7 receptors leads to a direct stimulation of glutamate release (Gray et al., 1996; Mansvelder and McGehee, 2000). Support for modulatory effects of presynaptic nAChRs activation in the PFC comes from a variety of approaches including electrophysiological recordings and assay of release from isolated nerve terminals (Dickinson et al., 2008; Lubin et al., 1999; Wang et al., 2006). A recent study testing the relative contribution of β2* nAChRs vs. α7 nAChRs on glutamatergic synaptosomes from PFC (Dickinson et al., 2008) demonstrated that both α7 and non-α7 nAChRs appear to be important although each modulates excitatory amino acid (EAA) release via distinct mechanisms. Taken together, these data suggest that nicotinic receptors activation selectively increases activity of inputs from the thalamus to the cortex over other glutamatergic synapses. Understanding how nAChRs can affect the function of cortical pyramidal neurons is essential to understanding nicotine's effects on cognition.

However, the task is significantly more complicated. While so many aspects of pyramidal cell function are well described, nAChRs are rarely found on pyramidal cells in the cortex (Gil et al., 1997; Nicoll et al., 1996; Porter et al., 1999; Xiang et al., 1998b). A recent study has found nAChRs on layer VI pyramidal cells (Kassam et al., 2008a). This specific population represents pyramidal cells projecting back to the thalamus. Despite the fact that nAChRs are rarely found on pyramidal cells, nicotine can still affect their function in many ways. In addition to glutamatergic inputs to layer V pyramidal neurons, glutamatergic inputs to several types of layer V interneurons were also excited by nicotine with a similar pharmacological profile (Couey et al., 2007). Although it was not shown in the study, it is tempting to speculate that these nicotine-sensitive glutamatergic inputs to interneurons were of thalamic origin, but this awaits further testing. Nicotinic regulation of excitatory inputs to inhibitory interneurons could serve to balance excitation and inhibition in the prefrontal cortex, which is thought to be crucial for cortical functioning and information processing (Markram et al., 2004). In this thesis, I have identified a novel group of PFC pyramidal neurons that express nicotinic receptors (Chapter 3).

10. Cortical interneurons and nicotinic actions

Interneurons form a more highly diverse group of nerve cells then pyramidal neurons and their nomenclature is still under debate. Probably more than 20 different

types of interneurons exist in the neocortex and can be classified based on their different morphology, the expression of distinct cellular markers and differences in electrophysiological properties. Genetic tools allow us to study defined neuronal populations in an unbiased way, reproducible from laboratory to laboratory and give a unique opportunity to manipulate these neurons in neuronal circuitries. However, firing profiles prove to be still useful though to broadly classify interneurons into groups that partly overlap with the expression of cellular markers and morphology (DeFelipe et al., 2013). Inhibitory neurons can also be directly excited by nicotinic receptors. At least two types of interneurons are recognized to be morphologically and functionally distinct classes: fast spiking cells (FS) and low-threshold spiking cells (LTS) (Gupta et al., 2000; Kawaguchi, 1993; Kawaguchi and Kondo, 2002; Kawaguchi and Kubota, 1997; Markram et al., 2004; Wang et al., 2002). Fast spiking cells (FS) are physiologically equipped for high frequency firing, show little adaptation, and have been shown to synapse on or near the somata of their target cells (Gonzalez-Burgos et al., 2005; Kawaguchi and Kondo, 2002). As such, they occupy an ideal functional and morphological position to regulate the input window of pyramidal cells (Klyachko and Stevens, 2006). At least one study in the somatosensory cortex has demonstrated this functional position for FS cells (Sun et al., 2006). Their functional role also appears to extend to regulating plasticity in this microcircuit (Bacci and Huguenard, 2006). This association with thalamic inputs has also been confirmed in the PFC (Rotaru et al., 2005). While there is some disagreement as to whether FS cells express nAChRs, this discrepancy appears to be species specific. Studies in rodents have failed to find nAChRs on FS cells in the cortex (Couey et al., 2007b; Gulledge et al., 2007a). In contrast, in at least one study, FS cells in human cortex appear to express nAChRs (Krenz et al., 2001). FS cells appear to be important in regulating the precise timing of information coming into the cortex, and there is emerging consensus evidence that LTS interneurons play a role in shaping feedforward inhibition between excitatory cells (Kapfer et al., 2007; Silberberg and Markram, 2007). Like FS cells, LTS cells target specific dendritic subdomains of their target pyramids. In contrast to FS cells which are thought to regulate target cell activation and activity, LTS cells appear to regulate specific inputs to pyramidal cell apical dendrites, as well as mediating intralaminar feedforward inhibition in the cortex. These interneurons express large nicotinic currents, and excitatory input to these cells is also enhanced by nicotinic receptor stimulation (Couey et al., 2007; Gulledge et al., 2007). A third class of interneurons, identified based on their firing properties in response to depolarizing current steps, regular-spiking non-pyramidal neurons, were also excited by nicotine (Couey et al., 2007).

Despite more than a decade of studies to nicotinic receptor modulation of cortical microcircuit function, this picture is still incomplete. As described, the different layers of the PFC process different information streams. Distribution of nicotinic receptor expression also differs across cortical layers. Questions that remain include how

different layers of the PFC are modulated by nAChRs, how nAChR stimulation leads to activation of the prefrontal cortex and whether this is layer-specific. In chapter four we investigate therefore the synaptic mechanisms underlying nicotinic receptor modulation of the prefrontal cortex. We show that nAChR modulation of inhibition, in contrast to pyramidal neuron modulation, is a ubiquitous mechanism across cortical layers.

11. Nicotinic receptors and synaptic plasticity

The brain continuously adapts to the needs of the environment. Networks can change their connectivity to fulfill a new requirement because they are plastic. This makes us able to learn and memorize, but also to overcome brain injuries and neurological disease. Synapses change on short-time scales (short-term plasticity) and over longer periods (long-term plasticity). In addition, new synapses can be formed or old ones eliminated to change network properties (structural plasticity) (Holtmaat and Svoboda, 2009) and neurons change their properties in response to various chemicals (pharmacological plasticity). Neurons can also change the weight of many synapses at once (homeostatic plasticity) (Turrigiano and Nelson, 2004) and regulate the ability to undergo long-term potentiation or depression (metaplasticity) (Abraham, 2008). Spike-timing-dependent plasticity is a form of associative plasticity in which synapses change weight depending on the relation between the input that they receive and the output that they send away. In principal this relation is bidirectional and depending on the order. If a glutamatergic input precedes an action potential synapses are strengthened (LTP) and if it follows an action potential it leads to depression of synapses (LTD). Because of this associative relationship it is a popular cellular model for learning and memory (Feldman, 2012).

Nicotinic receptor activation directly changes hard-wired circuits by altering the membrane potential of the cell. On longer-time scales stimulation of nAChRs can lead to permanent changes in synaptic strength altering information transfer in glutamatergic synapses (Mansvelder et al., 2009). Depending on the location and subunit there is a wide diversity of effects of nicotine receptors on plasticity. In the hippocampus α7 nAChR activation induces calcium release from internal calcium stores to synchronize vesicular release and increase synaptic strength presynaptically (Sharma et al., 2008). In the ventral tegmental area presynaptic activation of the same receptor in combination with postsynaptic depolarization leads to long-term strengthening of glutamatergic synapses which depends on NMDA receptor activation (Mansvelder and McGehee, 2000). In vivo potentiation of these glutamatergic synapses onto the VTA might underlie rewarding properties of nicotine (Caille et al., 2009). In the hippocampus CA1 pyramidal neurons express α7 nAChRs postsynaptically which, when activated, enhance high frequency induced plasticity in the Schaffer collateral pathway (Ji et al., 2001). However, stimulating nAChRs on interneurons

reduces synaptic plasticity in the same protocol. Hence depending on the neuron type stimulated by nAChRs effects on synaptic plasticity differ (Ji et al., 2001). Endogenous cholinergic activation of nicotinic receptors has been shown to play an important role in orchestrating hippocampal plasticity (Gu et al., 2012; Gu and Yakel, 2011).

The role of synaptic plasticity in cognitive behaviors like attention is less clear. Prefrontal cortical synapses are plastic and change their strength during working memory related tasks (Laroche et al., 2000). Changes in strength of cortical glutamatergic synapses are thought to depend on the precise timing of pre- and postsynaptic activity and therefore named spike-timing dependent plasticity (Bi and Poo, 1998; Markram et al., 1997). The relative timing of pre- and postsynaptic plasticity results in specific postsynaptic changes in calcium concentration that determine whether strength will increase or decrease (Sjostrom and Nelson, 2002). The question whether nicotinic receptors in the prefrontal cortex affect STDP has not been answered. Since nicotinic receptors have a strong influence on inhibition, which is important regulator of synaptic plasticity and dendritic signaling (Meredith et al., 2003; Murayama et al., 2009), they might have a big impact on STDP rules. In chapter 4 Couey et al. investigate this question.

12. Nicotine and its short-term effects on attention

It has long been recognized that nicotine, the addictive substance in cigarettes, can have stimulating effects on brain function. The link to the psychoactive effects lies in the fact that nicotine stimulates nAChRs that are normally activated by the endogenous neurotransmitter ACh and thereby interfere with cholinergic signaling. By boosting signal-to-noise ratio, the cholinergic system in the brain is important for a variety of cognitive functions, such as learning, memory and attention processes that involve many different brain regions (Everitt and Robbins, 1997). As described above, activation of nAChRs can affect cognitive functions and, in particular, attentional performance. With a few exceptions, nAChRs agonists enhance cognitive performance, while antagonists have the adverse effect (Levin et al., 2006). Most clear effects are found on memory processes. For instance, nicotinic agonists improve working memory function (Levin et al., 1997) and overcome deficits induced by lesioning cholinergic innervation of the hippocampus. In contrast, nicotinic antagonists for different nicotinic subtypes applied to the hippocampus impair working memory function in the radial arm maze (Felix and Levin, 1997). Nicotine can also affect attention performance and some aspects of this appear to rely particularly on nicotinic receptor signalling in the prefrontal cortex (Hahn et al., 2003). Also in humans, nicotine was shown to change activity in the prefrontal cortex (Lawrence et al., 2002). In non-smoking human subjects, however, nicotine most often does not improve attention behavior (Counotte et al., 2013; Newhouse et al., 2004). Beneficial effects are mostly seen in smokers suffering from abstinence or

patient populations (Counotte et al., 2013; Newhouse et al., 2004b). In rodents, the enhancement of attention performance by nicotinic agonists was shown in a number of studies (Hahn et al., 2002, 2003b; Levin et al., 2006; Mirza and Bright, 2001; Mirza and Stolerman, 1998), but the effects reported were usually small, studied in rats and not found in all strains. Other studies have shown the absence of an effect or decrease in attention performance upon exposure to acute nicotine and shown mostly in mice (Bailey et al., 2010; Hahn and Stolerman, 2002; Pattij et al., 2007). Very little is known about the mechanisms underlying nicotine's effect on attention. On the short-term nicotine activates nAChRs and alters PFC network function (Chapter 4)(Couey et al., 2007). However, during smoking of a single cigarette nicotine concentrations remain elevated (Matta et al., 2007) and this is known to desensitize nAChRs on neurons (Mansvelder and McGehee, 2002; Pidoplichko et al., 1997). Whether desensitization plays a role in the prefrontal cortex is not known and is the main topic of chapter 5.

13. Long-term consequences of nicotine exposure during adolescence

Through the mechanisms discussed above, nicotinic AChR activation directly affects activity in cortical circuits involved in cognition. However, these same cellular and synaptic mechanisms also affect long-term synaptic plasticity, the effects of which outlast nAChR activation (Couey et al., 2007; Ji et al., 2001; Mansvelder and McGehee, 2000). Thereby, nicotine may exert lasting effects on cognition. Thus far, a very limited number of studies have addressed this hypothesis, and mechanisms underlying long-term effects of nAChR activation have not been addressed. The majority of adult smokers started the habit during adolescence (Breslau and Peterson, 1996; Leslie et al., 2004), and an ever-growing amount of evidence shows that nicotine exposure during adolescence not only has direct effects on prefrontal cortical function but can also lead to adaptations in this brain area that last into adulthood.

Adolescent smoking strongly correlates with cognitive and behavioral impairments during later life (Chambers et al., 2003; Ernst et al., 2006; Wiers et al., 2007). Functional MRI studies show that during working memory and attention tasks adolescent smokers have reduced PFC activation, less efficiency and altered functional coordination when compared to abstinent adolescents (Jacobsen et al., 2007; Musso et al., 2007). Importantly, the history of smoking duration in years is correlated with the extent of diminished PFC activity, suggesting that nicotine exerts long-lasting effects on PFC function (Musso et al., 2007). Though studies in humans reveal strong correlations between adolescent smoking and cognitive impairments during later life, genetic variability and diverse social environment make it almost impossible to disentangle the causal relationships. Animal models with a highly uniform genetic and environmental background between individuals offer the opportunity to directly address lasting prefrontal adaptations in response to nicotine exposure. In rodents, nicotine exposure during adolescence induces stronger changes in gene expression

in the PFC than during other periods of development and adulthood (Polesskaya et al., 2007; Schochet et al., 2008; Schochet et al., 2005). In PFC after chronic nicotine treatment, the maximal regulation of genes involved in vesicle release, signal transduction, cytoskeleton dynamics and transcription was observed at postnatal day 35, suggesting the role of nicotine in initiating long-term structural and functional adaptations in adolescent PFC (Polesskaya et al., 2007). The activity of specific early response genes (arc) was found to be elevated in adolescent PFC after acute nicotine exposure (Schochet et al., 2008). In addition, c-fos expression in the PFC in response to nicotine exposure is maximal during adolescence (Leslie et al., 2004). The expression of key molecules involved in plasticity is also altered in the PFC by adolescent nicotine exposure. Acute nicotine induces increases in the expression of the dendritically targeted dendrin mRNA in PFC of adolescent but not adult animals. Dendrin is an important component of cytoskeletal modifications at the synapse and therefore can lead to unique plasticity changes in the adolescent PFC (Schochet et al., 2008). Lasting synaptic adaptations involve activation of intracellular signalling pathway and such enzymes as extracellular regulated protein kinase (ERK) and cAMP response element binding protein (CREB). Specifically in the PFC, increases in phosphorylation of both these enzymes were found after repeated nicotine exposure (Brunzell et al., 2003).

Does adolescent nicotine exposure result in lasting altered cognitive function? Recently, this question was addressed by Counotte et al. (Counotte et al., 2009). Rats were trained in the 5 choice serial reaction time task and were injected with either nicotine or saline for 10 days during adolescence (postnatal days 34 – 43) and attentional performance was tested 5 weeks after the animals received the last injection with nicotine. Animals that received nicotine during adolescence showed a doubling in premature responses and a reduction in correct responses, suggesting increased impulsive behavior and reduced attentional performance. Animals that received nicotine as adults did not show changes in impulsivity nor in attention performance (Counotte et al., 2009). Recent studies uncovered some important mechanisms underlying network adaptation in exposure to nicotine and ultimately attention behavior (Counotte et al., 2012b; Goriounova and Mansvelder, 2012a). After nicotine exposure, nicotinic receptors are upregulated and show enhanced sensitivity to stimulation by agonists (Counotte et al., 2012a). The increased number of receptors declines into adulthood, but leads to reduced levels of the mGluR protein. Diminshed levels may explain the lasting effects of nicotine on attention described above as the attentional deficits are ameliorated by mGluR stimulation (Counotte et al., 2011). Reduced mGluR function leads to altered information processing in the PFC. Short-term plasticity rules as well as spike-timing dependent plasticity rules are altered. After nicotine treatment during adolescence timed LTP is easier to induce (Goriounova and Mansvelder, 2012b). Also short-term depression is less prominent, showing that synapses filter information differently after nicotine exposure (Counotte et al., 2011). These mechanisms could underlie altered attention behavior in adult rats treated with

nicotine during adolescence. What the initial mechanisms are through which nicotine alters information processes in the cortex is less clear. Since nicotinic receptors are highly upregulated after nicotine exposure we hypothesized that desensitization plays an important role in the PFC. We set out to test this hypothesis in chapter 5 of this thesis.

Conclusions

Cholinergic signaling in the prefrontal cortex is crucial for attentional performance. Acetylcholine signaling in the prefrontal cortex takes place on multiple timescales to facilitate cue-detection. How cholinergic signals in the brain are translated into functional signals that mediate attention is not understood. In particular, which receptors subtypes play a role in attention and in which brain area they exert their action remains elusive. The fast dynamics of cholinergic signaling seen during cue-detection suggests fast ionotropic nicotinic receptors play a role in translating cholinergic signaling into functional output of the prefrontal cortex. Genetically modified mouse models offer the opportunity to study the role of nicotinic receptor subtypes in attention and their role in altering information transfer in the prefrontal cortical network. Nicotinic receptors are found on interneurons as well as pyramidal neurons in the PFC. Therefore they can alter information processing in the PFC by altering excitatory as well as inhibitory transmission, but how these mechanisms differ across cortical layers and how this influence plasticity rules is not known. Nicotine acts on the cholinergic system and influences attention behavior in the short- and long-term. Nicotine can influence information processing by first activating nicotinic receptors and subsequently desensitizing them, thereby interfering with cholinergic signaling. How this affects functioning of prefrontal cortical microcircuits is investigated in this thesis.

Synopsis of the thesis

The main aim of the research projects carried out in this thesis is to (i) elucidate the contribution of different nicotinic acetylcholine receptor subtypes to attention behavior, (ii) investigate how these different subtypes alter the function of microcircuitries in the medial PFC when activated by acetylcholine and (iii) how the function of these receptors and circuits changes when exposed to nicotine.

In **chapter 2** we ask the question whether α7 and β2* nicotinic receptors are involved in regulating attentional behavior. We use the 5-choice serial reaction time task as a paradigm to assess differences in attention behavior. Mice deficient for β2 subunits were found to have a prominent attention deficit as reflected by an increased percentage of omissions. Rapid increases in cholinergic signaling in the mPFC are seen during cue-detection. Therefore we next asked whether the attentional deficit

could be explained by the absence of β2* nicotinic receptors in the prelimbic cortex. To address this question we use single gene targeting with lentiviral vectors to restore functional receptors in the prelimbic network. We show a causal role of β2 subunits in the prelimbic cortex in attention. Moreover, re-expression in this area is sufficient to fully rescue attention deficits displayed by mice deficient for β2 subunits.

The findings in chapter 2 pose the question how nicotinic receptors alter information processing in the prelimbic cortex. The cortical layers of the prefrontal cortex form distinct microcircuits and their output is send to different areas in the brain. I hypothesize that nicotinic activation of the prefrontal cortical network is layer-specific. In **chapter 3** I test this hypothesis and investigate the role of different nicotinic receptor subunits in activating the PFC. Using patch-clamp whole-cell recordings we show that nicotinic receptors can activate inhibitory as well as excitatory neurons. Interneurons modulation by nicotinic receptors is a ubiquitous mechanism across cortical layers, whereas pyramidal neuron activation is layer-specific. It is hard to predict how stimulation of these opposing cell types balances out to change the output of the PFC. Therefore we used an integrative two-photon network imaging to investigate this and test the hypothesis that prefrontal cortical activation is layer-specific.

The findings in chapter 3 show that an increase in inhibitory tone is a major effect of nicotinic receptor stimulation on prefrontal cortical function. Part of the interneurons we find to be stimulated by nicotinic receptors target dendritic compartments of pyramidal neurons. Hence this might alter dendritic computation. Therefore we hypothesize in **chapter 4** that an increase in inhibition hampers communication between soma and dendrite and alters the rules for inducing spike-timing dependent plasticity. We use extracellular stimulation to assess synaptic inputs to layer V pyramidal neurons and induce plasticity in the absence and presence of nicotine. We found nicotine to increase the threshold for induction of spike-timing dependent plasticity which depends on increased GABA release by nicotinic receptors which reduces backpropagating action potentials as revealed by two-photon imaging.

During adolescence the circuitry of the prefrontal cortex is highly sensitive to maladaptations when exposed to nicotine. Nicotine exposure leads to upregulation of receptors and alters protein expression levels. What the initial mechanisms are that might cause adaptation is not known. I hypothesize that smoking concentrations of nicotine strongly desensitize nicotinic receptors. In **chapter 5** I investigate this question by assessing nicotinic receptor currents on single cells. I find that nicotine strongly interferes with cholinergic signaling through β2* receptors, but not α7 receptors. The level of desensitization was layer and neuron type depended. Differential sensitivity of β2* nAChRs to nicotine might depend on the α5 subunit and I investigate this using knockout mice. In addition, we perform two-photon network experiments to investigate how nicotine shifts cholinergic activation of the PFC.

The experimental work described in this thesis offer insights in the role of nicotinic

receptors in attention and how they regulate the underlying cortical circuitry. In addition, it shows how nicotine can alter the function of these circuits. In **chapter 6** I conclude that β2* receptors have a central role in regulating attention. I place these findings in the context of how β2* nicotinic receptors regulate the PFC network, focusing on inhibition. By placing in it in the context of existing literature I argue how inhibition might regulate output of the PFC to limbic structures to control attention performance. Finally, I discuss how nicotine alters the function of the PFC on the short-term and discuss how desensitization of nicotinic receptors might lead to maladaptive changes in the PFC circuit causing reduced attention performance.

Nicotinic acetylcholine receptor β2 subunits in the medial prefrontal cortex control attention.

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Chapter 2

Published in Science 2011 Aug 12;333 12;333(6044):888-891

Abstract

More than one third of all people are estimated to experience mild to severe cognitive impairment as they age. Acetylcholine (ACh) levels in the brain diminish with ageing and nicotinic ACh receptor (nAChR) stimulation is known to enhance cognitive performance. The prefrontal cortex (PFC) is involved in a range of cognitive functions and is thought to mediate attentional focus. We found that mice carrying nAChR β2-subunit deletions have impaired attention performance. Efficient lentiviral vector-mediated re-expression of functional β2-subunit-containing nAChRs in PFC neurons of the prelimbic area (PrL) completely restored the attentional deficit, but did not affect impulsive and motivational behavior. Our findings show that β2-subunit expression in the PrL PFC is sufficient for endogenous nAChR-mediated cholinergic regulation of attentional performance.

Cortical ACh release from the basal forebrain is essential for proper sensory processing and cognition (Everitt and Robbins, 1997; Goard and Dan, 2009; Woolf and Butcher, 2010), and tunes neuronal and synaptic activity in the underlying cortical networks (Poorthuis et al., 2009; Steriade, 2004). Degeneration of cholinergic neurons during ageing and Alzheimer's disease results in cognitive decline, notably a loss of memory and the ability to sustain attention (Lawrence et al., 2002; McKhann et al., 1984). Interfering with the cholinergic system strongly affects cognition (Dalley et al., 2004; Everitt and Robbins, 1997; Hahn et al., 2003; Howe et al., 2010; Muir et al., 1995; Sarter et al., 2005; Stolerman et al., 1995). Rapid changes in prefrontal cortical ACh levels at the scale of seconds are correlated with attending and detecting cues (Parikh et al., 2007; Sarter et al., 2009). Various types of nAChR subunits are expressed in the PFC (Couey et al., 2007a; Kassam et al., 2008b; Lambe et al., 2003a), and in particular nAChRs containing β2-subunits are thought to enhance attention (Howe et al., 2010). However, the causal relationship between nAChR β2-subunits (henceforth β2*-nAChRs) expressed in the medial PFC (mPFC) and attention performance has not yet been demonstrated.

We first determined whether absence of nicotinic β2-subunits affects attentional behavior in the 5-choice serial reaction time task (5-CSRTT), a well-established test set-up that taxes various aspects of attentional control over performance (Robbins, 2002). Mice lacking β2-subunits of nAChRs (β2-/-), and their wild-type littermates (WT) were trained to detect and respond to a brief light stimulus randomly presented in one of five nose poke holes to receive a food pellet. β2-/- mice showed normal locomotor activity in an open field test (Fig S1), normal sensorimotor gating in a pre-pulse inhibition test (Fig S2), and normal 5-CSRTT acquisition (Fig S3).

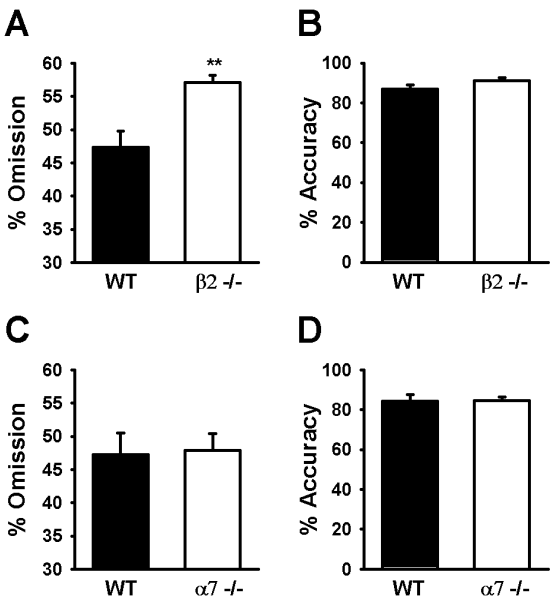

Figure 1. β2-nAChR subunit is necessary for normal performance in 5-CSRTT.
(A,B) Percentage omission (A) and accuracy (B) of WT (n = 15, black) and β2-/- mice (n = 14, white) during baseline training (SD1). ** p < 0.01, Newman-Keuls post hoc test. (C,D) Percentage omission (C) and accuracy (D) of WT (n = 12, black) and α7-/- mice (n = 25, white) during SD1. Data in all figures are shown as mean ± S.E.M.

After complete acquisition of the 5-CSRT task, animals were trained at the stimulus duration of 1 second (SD1) for 10 more days until they reached stable performance (Fig S4). Baseline 5-CSRTT performance was then calculated from the 6th until the 10th session at SD1 (Fig 1A,B). β2-/- mice exhibited significantly more omissions than their WT littermates ($F(1,27) = 12.45$; $p < 0.01$) (Fig 1A), whereas the level of accuracy was not significantly different ($F(1,27) = 2.56$; NS) (Fig 1B). We found no effect of genotype on any other measures, such as number of initiated trials ($F(1,27) = 1.99$; NS), number of premature responses ($F(1,27) = 0.003$; NS), correct responses latency ($F(1,27) = 2.03$; NS), or latency to collect earned food pellets ($F(1,27) = 0.12$; NS) (Table S1), suggesting that increased omission reflected impairments in stimulus detection processes in β2-/- mice rather than motor or motivational deficits. β2-/- mice and their WT littermates did not differ in the number of food pellets earned by responding to a single cue light, nor in the maximal number of responses in a progressive ratio for earning food pellets (Fig S5). In contrast to β2-/- mice, mice lacking α7-subunits of nAChRs (α7-/-) exhibited similar levels of omission ($F(1,35) = 0.10$; NS) and accuracy ($F(1,35) = 0.05$; NS) as their WT littermates (but see supplementary information; Fig 1C,D and Table S3). To further characterize attentional deficits, we compared performance in a variable stimulus procedure, in which stimulus durations were randomly decreased to 0.5 and 0.25 seconds (Fig S6 and Table S2). β2-/- mice made significantly more omissions than WT mice at every stimulus duration (Fig S6A and Table S2), but had similar accuracy and motivation to earn food rewards (Fig S6B and Fig S5), whereas no difference was observed between α7-/- and WT animals (Fig S6C,D and Table S4).

To further understand the specific role of β2-containing nAChRs in mediating the effects of endogenous acetylcholine on cognition (Granon et al., 2003), we selectively re-expressed the β2-subunit (Maruki et al., 2003) in the prelimbic area (PrL) of the mPFC of β2-/- mice. The mPFC is critically involved in attentional performance (Dalley et al., 2004). We re-expressed β2-subunits in combination with enhanced green fluorescent protein (eGFP) by injection of the β2-eGFP bi-cistronic vector (Avale et al., 2011; Maskos et al., 2005) into the PrL PFC of β2-/- mice (KOVEC). As a control, we used a lentiviral vector expressing eGFP only in β2-/- mice (KOeGFP) and WT littermates (WTeGFP). Coronal sections showing the site of lentivirus injection revealed that viral re-expression was selective to the PrL of the mPFC (Fig 2A). The efficacy of this *in vivo* re-expression strategy was demonstrated by confocal analysis showing that eGFP co-localized with a neuronal marker (NeuN) in KOVEC mice (Fig 2A), demonstrating efficient transduction of β2-eGFP vectors in PrL neurons. β2-subunits do not form functional nAChRs by themselves, but require nAChR α-subunits to co-assemble into functional receptors (McGehee and Role, 1995b). Therefore, in KOVEC mice not all eGFP expressing neurons will have β2*-nAChRs. Only in neurons that express nAChR α-subunits, lentivirus-mediated expression of β2-subunits will result in functional nAChRs containing β2-subunits. We thus made whole-cell recordings

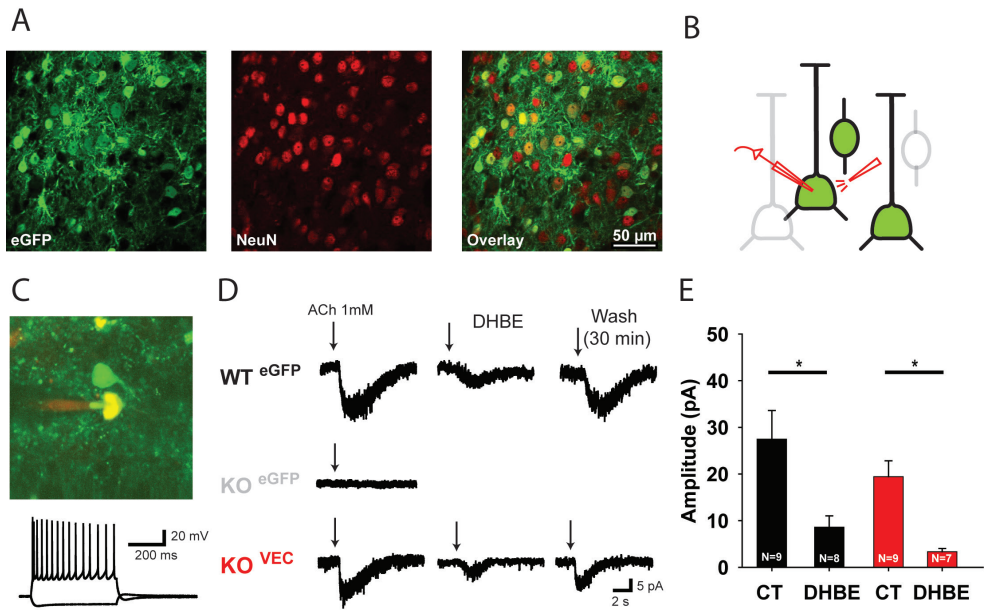

Figure 2. Lentiviral restoration of functional β2*-nAChRs in the mPFC.
(A) Coronal section (1.9 mm from bregma) showing the injection site in the prelimbic mPFC (left) and confocal images of acute coronal sections showing neuronal eGFP (green) expression (red, NeuN) in KOVEC mice and merged image (right).
(B) Experimental setup.
(C) Patched eGFP-positive neuron.
(D) Current traces recorded from WTeGFP (n = 9, black), KOeGFP (n = 9, grey) and KOVEC (n = 9, red) neurons. ACh was locally applied (1 mM, 100 ms) in control (left), in the presence of β2-containing nAChRs antagonist, DHβE 1 µM (middle), or after 30 min wash-out (right).
(E) Summary of ACh-induced inward currents for WTeGFP (black) and KOVEC (red). nAChR current amplitudes of WTeGFP and KOVEC neurons were not statistically different.

from eGFP expressing neurons in the three groups and tested their response to acetylcholine (ACh) (Fig 2B-D). In WTeGFP mice, locally applied ACh (1 mM) induced inward currents with slow kinetics, characteristic of β2*-nAChRs (Fig 2D). These currents were strongly reduced by the antagonist of β2*-nAChRs, dihydro-β-erythroidine (DHβE 1 µM; $t(7) = -3.15$; $p < 0.05$; Fig 2D,E). KOeGFP neurons never showed slow inward currents in response to ACh application (Fig 2D). Neurons in the mPFC of KOVEC mice showed slow inward currents reminiscent of functional β2*-nAChR responses (n = 9 of 17 EGFP positive neurons, Fig 2D). These currents were strongly reduced by DHβE ($t(6) = -5.02$; $p < 0.01$; Fig 2D,E), showing the successful re-expression of functional β2*-nAChRs in KOVEC mice.

We addressed the question whether β2*-nAChRs specifically in the PrL mPFC would be sufficient for optimal attentional performance. We therefore tested whether impaired performance of β2-/- mice was rescued by targeted re-expression of the β2-subunit in the PrL mPFC. Preliminary analysis before viral expression showed

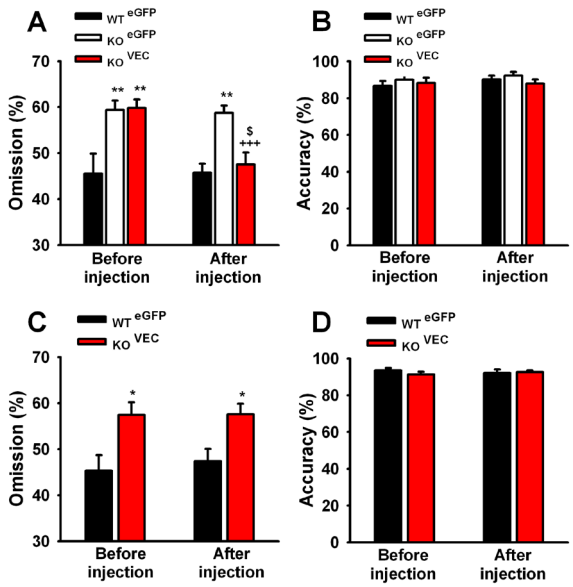

Figure 3. Targeted re-expression of β2-nACR subunits in PrL mPFC restores performance.
(A,B) Percentage omission (A) and accuracy (B) (SD1) before and after viral injection for WTeGFP (n = 11, black), KOeGFP (n = 11, white) and KOVEC mice (n = 11, red). ** $p < 0.01$, compared with WTeGFP; \$ $p < 0.05$, compared with KOeGFP; +++ $p < 0.001$, before and after virus injection, Newman-Keuls post hoc test.
(C,D) Re-expression of β2-nACR subunits in the anterior cingulate cortex did not restore attention performance. Percentage omission (C) and accuracy (D) (SD1) before and after viral injection for for WTeGFP (n = 11, black) and KOVEC mice (n = 4, red). * $p < 0.05$, compared with WTeGFP.

comparable findings between the independent batches of mice, with a significant increase in omissions in β2-/- mice (Fig S7). One week after virus introduction, WTeGFP, KOeGFP, and KOVEC mice were re-trained in the 5-CSRTT procedure using SD1 for 14 days before the effects of lentiviral intervention were assessed.

At the end of these 14 days, WTeGFP and KOeGFP animals performed at the same levels as they showed before virus injection, but KOVEC mice performed significantly better than before injection (Fig 3). The percentage of omission of the three groups of mice was differentially affected by lentivector injection (group effect: $F(2,30) = 10.73$; $p < 0.001$; injection time effect: $F(1,30) = 6.12$; $p < 0.05$; group × injection time interaction: $F(2,30) = 9.29$; $p < 0.001$) (Fig 3A). Although KOeGFP mice made more omissions than WTeGFP at each time point (WTeGFP vs. KOeGFP, $p < 0.01$), both groups exhibited the same percentage of omissions before and after virus injections (NS, eGFP), and hence were not affected by eGFP expression. Re-expression of β2-subunits in the mPFC (KOVEC) significantly decreased the percentage of omissions (KOVEC before vs. KOVEC after, $p < 0.001$). Moreover, the rescue in KOVEC mice was complete and these mice reached the same number of omission as WTeGFP mice (WTeGFP vs. KOVEC, NS), and made significantly less omissions than KOeGFP mice (KOeGFP vs. KOVEC, $p < 0.05$). This rescue-effect was selective for omissions since β2 re-expression had no significant effect on accuracy (group effect: $F(2,30) = 1.92$; NS; injection effect: $F(1,30) = 2.42$; NS; group × injection interaction: $F(2,30) = 2.36$; NS) (Fig 3B), or any other measures (Table S5). This rescue-effect was also observed during a variable stimulus procedure (Fig S9), as well as during a variable inter-trial interval (ITI) procedure in which the stimulus presentations were temporally unpredictable (Fig S10), further supporting the fact that β2-subunit restoration in the PrL is

sufficient for proper attention performance. A similar rescue-effect of β2 re-expression in KOVEC mice was observed in an independent group of animals (Fig S11). After these behavioral experiments, the mice were sacrificed and neuronal expression of eGFP and functional β2*-nAChRs in the PrL was confirmed. β2 subunit re-expression in the anterior cingulate had no effect on omission or accuracy (Fig 3C,D), in line with the finding that cholinergic projections to the anterior cingulate cortex are not involved in 5-CSRTT performance (Muir et al., 1996a).

Our findings show that expression of β2*-nAChRs is necessary for optimal attentional performance in mice and that restoring expression of β2*-nAChRs in the mPFC PrL area is sufficient for optimal performance. Nicotinic AChRs containing β2 subunits are located on cell bodies of neurons as well as on thalamocortical afferents in the PrL PFC (Couey et al., 2007a; Lambe et al., 2003a). The latter have also been suggested to be involved in attention and processing of sensory stimuli (Lambe et al., 2003a). The present study reveals that restoration of β2*-nAChR receptors, specifically in the PrL area of the mPFC, is sufficient to restore the attentional deficit of β2-/- mice to wild type levels. Attentional control therefore appears to be mediated by endogenous acetylcholine acting on β2*-nAChR receptors expressed by neurons located within the PrL mPFC, although a role for β2*-nAChRs on thalamic projections cannot be entirely excluded based on the present results. Nevertheless, the nAChR system in the PrL mPFC is a principal factor in attentional control. Consistent with this, rapid changes of ACh levels in mPFC are correlated with cue attending and detection (Parikh et al., 2007b), an effect mainly due to mPFC β2*-nAChRs stimulation (Parikh et al., 2010a). Our findings have implications relevant for understanding the neurobiology of attention and suggest agonists or positive allosteric modulators at these mPFC β2*-nAChRs within the PrL PFC as potential target for the development of more effective treatments for cognitive impairments.

The authors thank Jorn Staal and Hans Lodder. UM and ABS received funding from the EU 7th Framework Programme 'Neurocypres' (HEALTH-F2-2007-202088), HDM from NWO (917.76.360, 912.06.148) and the VU University board (VU Stg ERC).

Supporting material

Subjects

β2 knockout male mice (n = 42) and their wildtype littermates (n = 40) and α7 knockout male mice (n = 25) and their wildtype littermates (n = 12) or 6-7 weeks old C57Bl/6J were used. They were housed individually in macrolon enriched cages under a regular 12-h light/dark cycle at controlled room temperature (21 ± 2°C) and humidity (60 ± 15%). Animals were maintained at approximately 85% of their free-feeding weight, starting 1 week prior to the beginning of the experiments by res-

tricting the amount of standard rodent food pellets. Water was available ad libitum. All experiments were conducted with the approval of the animal ethical committee of the VU University, Amsterdam, the Netherlands.

Lentiviral expression vector and stereotaxic procedure

Vectors were based on the previously described pTRIPΔU3 (1). The re-expression lentivector is a bicistronic β2-IRES2-eGFP construct, previously described (2). Briefly, the mouse phosphoglycerate kinase (PGK) promoter was PCR amplified from a PGK-nls lacZ expression vector, M48, and ligated into pTRIPΔU3. To generate the β2- IRES2-eGFP construct a cloning site was created in the pIRES2-EGFP expression plasmid by mutagenesis, and the wild-type mouse b2 subunit, containing a consensus Kozak translation initiation site, was then ligated into plasmid pIRES-EGFP. The β2-IRES2-eGFP cassette was then ligated into the pTRIPΔU3-PGK vector using XhoI-SalI sites. Finally, the WPRE sequence was added. The PGK-eGFP control lentivector, is identical to the bicistronic version, but lacks the β2-IRES2 portion. Lentiviral particles were generated as previously described (2). Mice aged 8-10 weeks were anesthetized (isoflurane) and introduced into a stereotaxic frame adapted for mice. Lentivector (2 µl at 50 ng p24 protein per µl) was injected bilaterally in the mPFC (anteroposterior +1.9 mm; lateral, ±0.5 mm from bregma and −2.5 mm from the surface of the skull) (Paxinos and Franklin, 2001). After one week of recovery, mice were re-trained in the 5-CSRTT procedure using SD1 for 14 days.

Immunohistochemistry

To determine the expression pattern of the β2-eGFP bi-cistronic vector, KOVEC injected mice were anesthetized and perfused transcardially with PBS followed by 4% paraformaldehyde (PFA) dissolved in PBS. Brains were removed and post-fixed in 4% PFA overnight at 4°C and subsequently in 30% sucrose dissolved in PBS (~48hr at 4°C). Coronal brains (35 µm) were collected and washed in PBS, treated with 0.2% Tween 20 (PBS-T), immerged in a 5% normal goat serum blocking solution, and incubated overnight with primary antibodies against GFP (1:2000) and NeuN (1:1000) followed by incubation with secondary antibodies conjugated to Alexa Fluor 488 (green, 1:400) and Alexa Fluor 568 (red, 1:400). Transduction efficienty was quantified using a confocal microscope by comparing the GFP cells with either NeuN, CAMKII or GAD76 immunoreactive cells.

Electrophysiological recordings

Slice preparation

At the end of the behavioral experiments, animals were sacrificed and medial prefrontal (mPFC) coronal slices (250 µm) were prepared. Brain slices were prepared in ice-cold artificial cerebrospinal fluid (ACSF), which contained: 125 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 7 mM MgSO4, 0.5 mM CaCl2, 26 mM NaHCO3, and 10

mM glucose (300 mOsm). Slices were then transferred to holding chambers in which they were stored in aCSF, which contained the following: 125 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 2 mM MgSO4, 1 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose, bubbled with carbogen gas (95% O2/5% CO2). Slices were left to recover at room temperature for one hour.

Two-photon imaging

Two-photon imaging was performed to visualize GFP positive cells and to target them for patch-clamp recordings with Alexa 594 containing pipettes. This was done on a multibeam two-photon laser scanning microscope system coupled to a Ti:Sapphire laser (excitation at 840nm) and two PMTs located behind a dichroic beamsplitter with an edge at 562nm. Excitation light was blocked using a 750nm low-pass filter. In addition, there was a dichroic mirror transmitting wavelengths above 800nm for excitation and reflecting it to the PMTs for wavelengths below 800nm. The objective used had a 20X magnification and a 0.95 numerical aperture.

Patch-clamp recordings

Recordings were made using Multiclamp 700B amplifiers, sampled at a frequency of 20 kHz, digitized by the pClamp software, and later analyzed off-line using Matlab. Patch pipettes (4–6 MOhms) were pulled from standard-wall borosilicate capillaries and were filled with intracellular solution: 140mMK-gluconate, 1mMKCl, 10mM-HEPES, 4mMK-phosphocreatine, 4 mM ATP-Mg, and 0.4 mM GTP (pH 7.2–7.3, pH adjusted to 7.3 with KOH) (290–300 mOsm). Action potential profiles of cells were made using hyperpolarizing and depolarizing current steps. Nicotinic receptor currents were tested by pressure ejection of acetylcholine (Ach) for 100 ms using a Picospritzer III from a glass electrode with a tip opening of ~ 1 µm. The presence of atropine (200 nM) prevented stimulation of muscarinic receptors and during most of the experiments DNQX (10 µM) and bicuculline (1 µM) were used to block synaptic transmission. All experiments were performed at 31-34º C.

Given the prominent β2 nAChR current in PFC layer 6 pyramidal neurons (3), the whole cell recordings were biased to layer 6 neurons. Identification of neurons was based on morphological appearance of the soma in DIC microscopy as well as action potential firing in response to step depolarizations, but not for all neurons we were able to obtain sufficient morphological and physiological parameters to unambiguously identify them.

Operant response tasks: Apparatus and 5-CSRTT

Experiments were conducted in sixteen identical mouse five-hole nose poke operant chambers with stainless steel grid floors housed in sound-insulating and ventilated cubicles. Set in the curved wall of each box was an array of five circular holes, each equipped with an infrared detector and a yellow light emitting diode stimulus

light and a white house light mounted in the center of the roof. Rodent dustless precision pellets could be delivered at the opposite wall through a pellet dispenser. Animals were tested once daily from Monday until Friday.

During the initial shaping sessions, mice were trained to collect food pellets without any response requirement, and then to nose poke into one of the five holes to obtained food. During the 5-CSRTT sessions, each trial started with the illumination of the stimulus light in one of the holes (in pseudorandom order) for a restricted stimulus duration (SD) or until a response was made. Animals had to respond during the presentation of the stimulus light or within a limited hold of 4 seconds after termination of the presentation of the cue. A 5-CSRTT session terminated after a maximum of 60 delivered pellets or 25 min, whichever came first. Nose poking into the illuminated hole was considered a correct response and followed by the delivery of a food pellet and the start of 5 seconds intertrial interval (ITI), during which the stimulus light was turned off. A response into a non-illuminated hole was considered an incorrect response, extinguished the stimulus light and did not result in delivery of a food pellet. If an animal did not respond in any of the holes during stimulus presentation or the limited hold, an omission was counted. Both incorrect responses, omissions and responses during the ITI resulted in a time-out period of 5 seconds, during which the house light was turned off. In the first 5-CSRT task session the SD was set at 16 s, which was decreased in subsequent sessions to 8, 4, 2, 1.5 and 1 s if the subject reached criterion performance (omissions < 30%, accuracy > 60%, started trials > 50) or after a maximum of 10 consecutive sessions at the same stimulus duration, whichever came first. Thereafter, animals were trained at the stimulus duration of 1 second (SD1) for 10 more days. Baseline 5-CSRTT performance was calculated from the 6th until the 10th session at the stimulus duration of 1 second. Then after, effects of parameter manipulation were investigated by shortening the stimulus duration in the same session from 1 to 0.5 and 0.25 seconds. Task performance was reflected in the following behavioral measures: (1) percentage of omission calculated as [number of omitted trials / total trials] × 100; (1) accuracy, i.e. percentage of correct responses calculated as [number correct trials/(correct + incorrect trials)] × 100; (3) trial number; (4) premature responding, (5) correct response latency and (6) reinforcer latency.

Fixed and progressive ratio schedules of reinforcement

A separate cohort of mice was trained similar to the initial 5CSRT phase to respond into an illuminated response hole to obtain food, during sessions that ended after 25 min or 50 earned food pellets. After mice consistently initiated 50 trials each session, one session was administered in which all stimuli were presented in the same response hole (center hole) without limited hold. During this 30 minute session, mice were allowed to earn an unlimited number of food pellets under a fixed ratio 1 schedule (FR1) of reinforcement. Subsequently, mice were subjected to three 30 min sessions under a progressive ratio 2 (PR2) schedule of reinforcement.

Supplemental results

No impairment in locomotor activity

A one-way ANOVA performed on total distance travelled in the open-filed showed no significant effect of genotype ($F(1,27) = 0.77$; NS), indicating that β2-/- mice exhibited normal locomotor activity (Fig S1).

No impairment in sensorimotor gating

A two-way ANOVA performed on the percentage of pre-pulse inhibition showed a significant effect of pre-pulse intensities ($F(4,100) = 66.42$; $p < 0.001$), but no significant effect of genotype ($F(1,27) = 0.002$; NS) and significant interaction between pre-pulse intensities and genotype ($F(4,100) = 1.04$; NS), indicating that β2-/- mice exhibited normal sensorimotor gating abilities (Fig S2).

No impairment in learning at early stages of the 5-CSRTT acquisition

A two-way ANOVA performed on the percentage of omission with stimulus duration (16, 8, 4, 2, 1.5 and 1 sec) and genotype (WT vs KO) as factors revealed a significant effect of stimulus duration ($F(5,135) = 128.04$; $p < 0.001$), indicating that the percentage of omission increased when the stimulus duration decreased (Fig S3A). Moreover, there was a significant effect of genotype ($F(1,27) = 10.34$; $p < 0.01$), as well as a significant interaction between stimulus duration and genotype ($F(5,135) = 3.84$; $p < 0.01$). Post-hoc analysis revealed that β2-/- mice exhibited more omissions than their wild-type littermates at the lover stimulus duration of 2, 1.5 and 1 second ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively), but not at earlier stages of the acquisition (SD16, SD8, SD4, NS each) indicating that β2-/- mice normal learning abilities during early stages of the 5-CSRTT training.

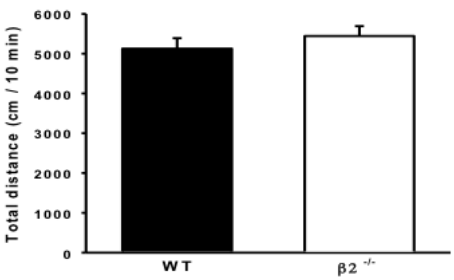

Figure S1. No impairment in locomotor activity.
A one-way ANOVA performed on total distance travelled in the open-filed showed no significant effect of genotype ($F(1,27) = 0.77$; NS), indicating that β2-/- mice exhibited normal locomotor activity (Fig S1).

Analysis performed on accuracy revealed a significant effect of stimulus duration ($F(5,135) = 55.03$; $p < 0.001$), indicating a progressive increase in accuracy until the mice reached stable performance (Fig S3B). However, there was no significant effect of genotype ($F(1,27) = 2.63$; NS), and no significant interaction between stimulus duration and genotype ($F(5,135) = 0.13$; NS), indicating no significant difference in accuracy during the acquisition of the 5-CSRTT task between the 2 groups of mice.

Stable level of performance and motivation during baseline SD1 training

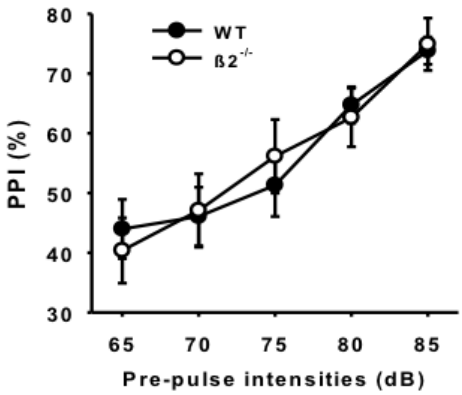

Figure S2. Deletion of β2-nACh subunit shows no impairment in sensorimotor gating abilities. Percentage of pre-pulse inhibition (% PPI) for WT (black) and β2-/- mice (white) at five different pre-pulse intensities (65, 70, 75, 80 and 85 dB). Data are presented as mean ± S.E.M.

After acquisition of the 5-CSRTT task, animals were trained at SD1 for 10 more days until they reached stable performance. Analysis of the omission rates at each daily SD1 session revealed a significant effect of genotype ($F(1,27) = 6.85$; $p < 0.01$), but no significant effect of day ($F(9,243) = 0.31$; NS) and no genotype by day interaction ($F(9,243) = 0.51$; NS) (Fig S4A), indicating that all mice have reached stable level of performance during the SD1. Mice have the tendency to decrease their responding as the session progresses, which could be an indication of fatigue and/or lost of motivation. To test whether β2-/- and WT mice showed a similar level of omission while the session progresses, we divided the total percentage of omissions into blocks of 20 trials (trials 1 to 20, 21 to 40 and 41 to 60) (Fig S4B). We found that mice make more omissions at the end of the session than at the beginning ($F(2,56) = 4.99$; $p < 0.05$; Newman-Keuls: Block 41-60 vs. Block 0-20; p Figure S3. < 0.01 and vs. Block 21-40; $p < 0.05$). However, there was no interaction between blocks and genotype ($F(2,56) = 0.34$; NS), indicating that β2-/- mice showed a similar decrease in performance across the session as WT animals. Thus, the increased omission observed in β2-/- mice cannot be due to an increased lost of motivation during the session compared to WT mice.

No impairment in motivation and satiety

We tested whether an increase in omission in β2-/- mice could be explained by a decreased motivation to earn rewards. Therefore we scored the total amount of earned sucrose pellets during a 30 minute session in which the mice needed to make one nose

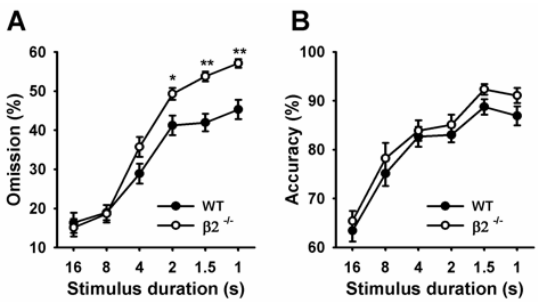

Figure S3. Deletion of β2-nACh subunit shows no impairment in learning during early stages of the 5-SCRTT acquisition. (A,B) Percentage of omission (A) and accuracy (B) of WT (black) and β2-/- mice (white) during the acquisition of the 5-SCRTT at different stages of training with a stimulus duration of 16, 8, 4, 2 and 1 second. Data shown are for the last 3 days of testing at each stimulus duration (mean ± S.E.M). * $p < 0.05$ and ** $p < 0.01$, significant difference between groups as revealed by the Newman-Keuls post hoc test.

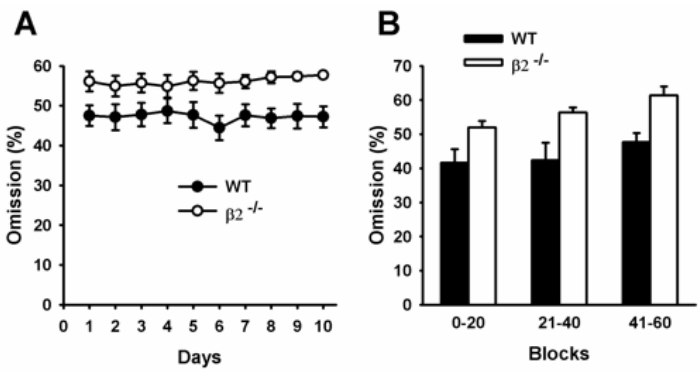

Figure S4. Stable level of performance and motivation during baseline SD1 training. (A) Percentage of omission of WT (black) and β2-/- mice (white) during the last 10 daily SD1 sessions. (B) Percentage of omission of WT (black) and β2-/- mice (white) during the SD1 sessions as a function of blocks of trials (block 1 to 20, 21 to 40 and 41 to 60 trials). Data are shown as mean ± S.E.M.

poke to earn one food pellet. β2-/- and WT mice earned the same amount of food pellets during the session, indicating that genotype had no effect on the motivation to earn rewards ($F(1,19) = 0.13$; NS, Fig S5A1). To test whether β2-/- and WT mice showed a similar motivation level while the session progresses, we divided the total session into 6 blocks of 5 minutes. Motivation to earn rewards decreased with block ($F(5,90) = 57.29$; $p < 0.001$, Fig S5A2). However, there was no significant effect of genotype ($F(1,18) = 0.11$; NS) and no interaction between genotype and motivation in different blocks ($F(5,90) = 1.36$; NS), indicating that β2-/- and WT have same levels of motivation during the whole task. To further test for motivational differences between the β2-/- and WT mice we next performed a progressive ratio test on three consecutive days. β2-/- and WT mice showed no difference in total amount of earned food pellets (Session 1: $F(1,19) = 0.12$; NS, Session 2: $F(1,19) = 0.01$; NS, Session 3: $F(1,19) = 1.58$; NS, Fig S5B1) and hence also no difference in the last completed ratio (Session 1: $F(1,19) = 0.12$; NS, Session 2: $F(1,19) = 0.01$; NS, Session 3: $F(1,19) = 1.58$; NS, Fig S5B2). Moreover, we found no difference of motivation during different blocks of single sessions (Session 1: $F(5,90) = 0.72$; NS, Session 2: $F(5,90) = 1.62$; NS, Session 3: $F(5,90) = 1.57$; NS, Fig S5B3-5).

β2-nACh subunit is necessary for normal performance of the mice during a variable stimulus procedure

The increase in attentional load yielded an overall increase in percentage of omissions in both WT ($F(2,54) = 15.73$; $p < 0.001$) and β2-/- mice ($F(1,27) = 34.62$; $p < 0.001$), indicating that omissions reflect an important aspect of attentional processes in both genotypes (Fig S6A). Further comparisons revealed that β2-/- mice made significantly more omissions than WT mice at every stimulus duration (SD1: $F(1,27) = 5.52$; $p < 0.05$, SD0.5: $F(1,27) = 9.97$; $p < 0.01$ and SD0.25: $F(1,27) = 12.95$; $p < 0.01$). Increasing attentional load by reducing the stimulus duration decreased accuracy ($F(2,54) = 14.04$; $p < 0.001$; Newman-Keuls : SD1 vs. SD0.5, $p < 0.01$; SD1 vs. SD0.25, $p < 0.001$; SD0.5 vs. SD0.25, $p < 0.01$) (Fig S6B and Table S2), but this

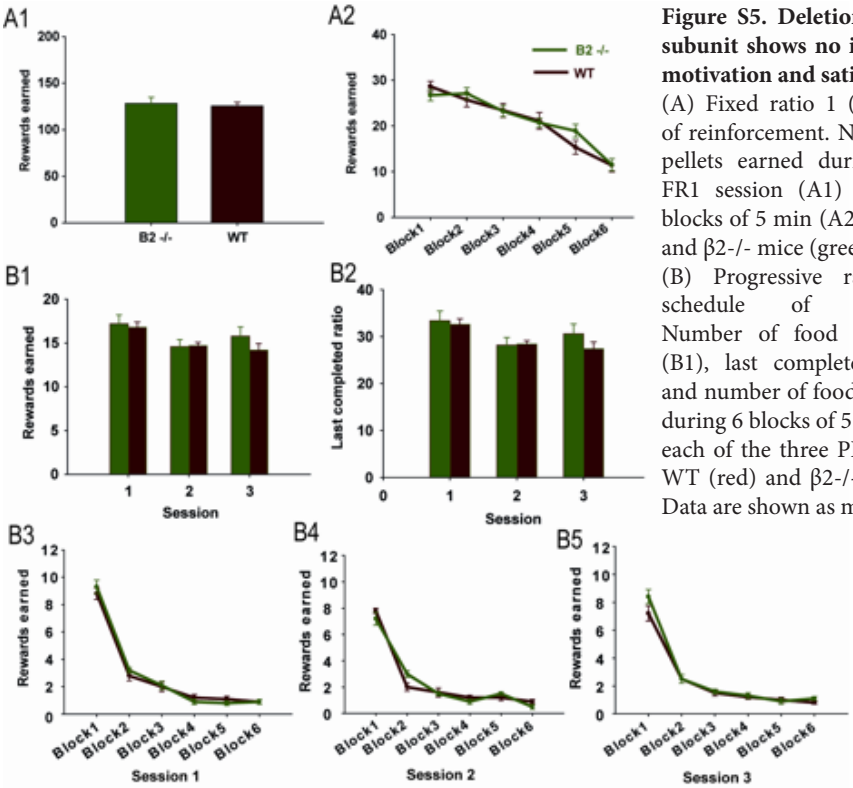

Figure S5. Deletion of β2-nACh subunit shows no impairment in motivation and satiety.
(A) Fixed ratio 1 (FR1) schedule of reinforcement. Number of food pellets earned during the entire FR1 session (A1) and during 6 blocks of 5 min (A2) for WT (red) and β2-/- mice (green).
(B) Progressive ratio 2 (PR2) schedule of reinforcement. Number of food pellets earned (B1), last completed ratio (B2), and number of food pellets earned during 6 blocks of 5 min (B3-5) for each of the three PR2 sessions for WT (red) and β2-/- mice (green). Data are shown as mean ± S.E.M.

effect was identical between the 2 groups of mice (genotype effect: $F(1,27) = 0.14$; NS; genotype × stimulus duration: $F(2,54) = 0.67$; NS).

No impairment of β2-/- mice in motor and motivational behaviors under the variable stimulus procedure

β2-/- showed a similar number of trials (genotype effect: $F(1,27) = 0.93$; NS; genotype × stimulus duration: $F(2,54) = 0.57$; NS), premature responses (genotype effect: $F(1,27) = 1.24$; NS; genotype × stimulus duration: $F(2,54) = 1.88$; NS), correct response latency (genotype effect: $F(1,27) = 0.92$; NS; genotype × stimulus duration: $F(2,54) = 0.55$; NS) and latency to consume the reinforcer (genotype effect: $F(1,27) = 2.07$; NS; genotype × stimulus duration: $F(2,54) = 0.38$; NS), indicating that increased omission in β2-/- mice was behaviorally specific and was not due to motor or motivational deficits (Table S2).

No impaired performance of α7 -/- in the 5-CSRTT

Under baseline training (SD1) (Fig 1C,D and Table S3), we found no significant difference between α7-/- mice and their WT littermates on the percentage of omission ($F(1,35) = 0.10$; NS), the percentage of accuracy ($F(1,35) = 0.05$; NS), number of

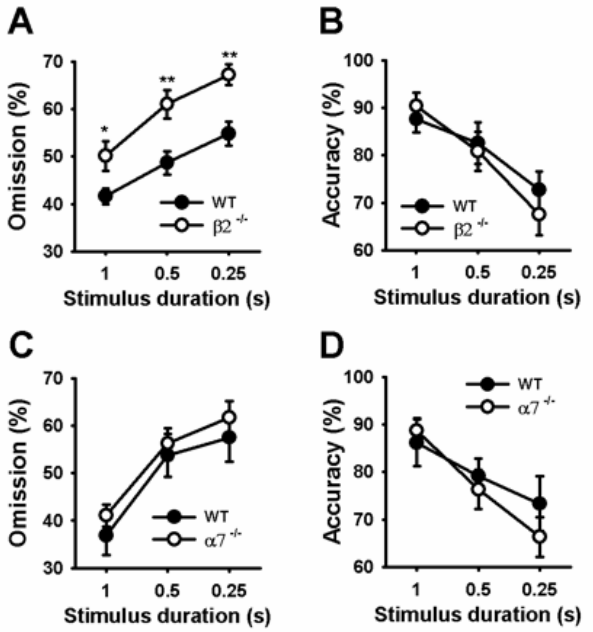

Figure S6. β2-nACh subunit is necessary for normal performance of the mice during a variable stimulus procedure in the 5-CSRTT.
(A,B) Percentage of omission (A) and accuracy (B) of WT (black) and β2-/- mice (white) during a variable stimulus procedure, in which stimulus duration was randomly decreased (1, 0.5 and 0.25 s). * $p < 0.05$ and ** $p < 0.01$, significant difference between WT and β2-/- mice as revealed by the Newman-Keuls post hoc test.
(C,D) Percentage of omission (C) and accuracy (D) of WT (black) and α7-/- mice (white) during a variable stimulus procedure. Data are shown as mean ± S.E.M.

initiated trials ($F(1,35) = 1.11$; NS), number of premature responses ($F(1,35) = 0.56$; NS), correct responses latency ($F(1,35) = 2.75$; NS), or latency to collect earned food pellets ($F(1,27) = 0.03$; NS). Reducing the stimulus duration within the same session (Fig S6C,D and Table S4) increased the number omission ($F(2,70) = 23.23$; $p < 0.001$; Newman-Keuls : SD1 vs. SD0.5, $p < 0.001$; SD1 vs. SD0.25, $p < 0.001$; SD0.5 vs. SD0.25, NS) and decreased accuracy ($F(2,70) = 11.75$; $p < 0.001$; Newman-Keuls : SD1 vs. SD0.5, $p < 0.01$; SD1 vs. SD0.25, $p < 0.001$; SD0.5 vs. SD0.25, $p < 0.01$). However, this effect was identical between the 2 groups of mice (genotype effect: $F(1,35) = 0.83$; NS; genotype × stimulus duration: $F(2,70) = 0.05$; NS for the omissions and genotype effect: $F(1,35) = 0.48$; NS; genotype × stimulus duration: $F(2,70) = 1.15$; NS for the accuracy). Moreover, α7-/- and WT mice showed a similar number of trials (genotype effect: $F(1,35) = 0.40$; NS; genotype × stimulus duration: $F(2,70) = 0.80$; NS), premature responses (genotype effect: $F(1,35) = 0.41$; NS; genotype × stimulus duration: $F(2,70) = 0.04$; NS), correct response latency (genotype effect: $F(1,35) = 1.49$; NS; genotype × stimulus duration: $F(2,70) = 0.67$; NS) and latency to consume the reinforcer (genotype effect: $F(1,35) = 1.29$; NS; genotype × stimulus duration: $F(2,70) = 0.77$; NS).

Corroboration of attention deficit in two independent experiments

The effects of absence of the nicotinic β2-subunit were assessed in an independent batch of animals (11 WT and 22 β2-/- mice). Analysis performed on the percentage of omissions showed a significant effect of genotype ($F(1,58) = 32.23$; $p < 0.001$), but no effect of experiments ($F(1,58) = 0.07$; NS) and no interaction between experiments

and genotype (F(1,58) = 1.90; NS) (Fig S7A). Analysis performed on accuracy revealed no effect of experiments (F(1,58) = 2.53; NS), no effect of genotype (F(1,58) = 2.27; NS), and no interaction between experiments and genotype (F(1,58) = 0.49; NS) (Fig S7B). Thus, the results were identical and animals reached the same levels of performance in the two experiments.

No effect of virus injection on locomotor activity

Analysis performed on the open-field showed that KOVEC travelled the same distance as WTeGFP and KOeGFP (F(2,20) = 0.53; NS), indicating that β2 subunit re-expression had no effect on locomotor activity (Fig S8).

No effect of virus injection on other measures in the 5-CSRTT under baseline condition (SD1) as control for motivation

The effect of lentivector injection (before vs. after injection) on other measures was first evaluated using SD1 (Table S5). Analysis revealed no effect of lentivector injection on the number of trials (group effect: F(2,30) = 1.0; NS; injection effect: F(1,30) = 3.7; NS; group × injection interaction: F(2,30) = 0.1; NS), number of premature responses (group effect: F(2,30) = 0.11; NS; injection effect: F(1,30) = 0.003; NS; group × injection interaction: F(2,30) = 0.007; NS), correct responses latency (group effect: F(2,30) = 0.29; NS; injection effect: F(1,30) = 0.86; NS; group × injection interaction: F(2,30) = 0.47; NS), or latency to collect earned food pellets (group effect: F(2,30) = 0.05; NS; injection effect: F(1,30) = 1.81; NS; group × injection interaction: F(2,30) = 0.19; NS).

Targeted re-expression of the β2-nACR subunit in the PrL area of the mPFC restores performance of the mice during a variable stimulus procedure

Under a variable stimulus procedure there was an overall increase in percentage of omissions (F(2,60) = 13.91; $p < 0.001$) (Fig S9A). Re-expression of the β2 subunit in the PrL (KOVEC) was sufficient to restore the performance of the β2-/- mice on omissions, as they reached similar performance as WTeGFP mice (F(2,30) = 3.62; $p < 0.05$; WTeGFP vs. KOVEC; NS) and made less omissions than the KOeGFP mice (KOVEC vs. KOeGFP; $p < 0.05$). β2 re-expression in the PrL had no significant

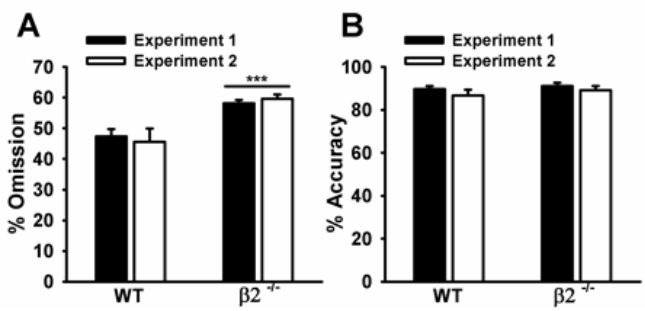

Figure S7. Corroboration of attention deficit in two independent experiments. Percentage of omission (A) and accuracy (B) of WT and β2-/- mice during the first (black) and the second (white) experiment. *** $p < 0.001$, significant difference between WT and β2-/- mice as revealed by Newman- Keuls post hoc test. Data are presented as mean ± S.E.M.

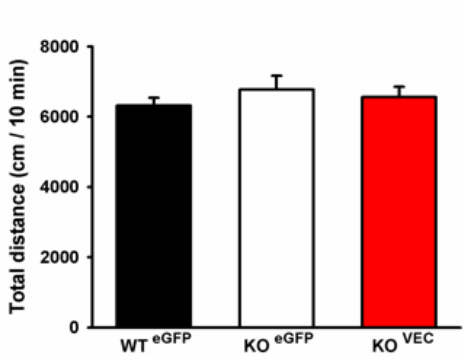

Figure S8. β2 subunit re-expression shows no impairment in locomotor activity.
Total distance travelled (cm) during a 10-min session in the open-field for WTeGFP (black), KOeGFP (white) and KOVEC (red) mice. Data are presented as mean ± S.E.M.

effect on accuracy (group effect: $F(2,30) = 0.75$; NS; SD effect: $F(2,60) = 13.49$; $p < 0.001$; group × SD interaction: $F(4,60) = 2.22$; NS) (Fig S9B), number of trials (SD effect: $F(2,30) = 1.0$; NS; group effect: $F(2,30) = 1.0$; NS; group × SD interaction: $F(2,30) = 1.0$; NS), number of premature responses (SD effect: $F(2,30) = 2.99$; NS; group effect: $F(2,30) = 0.84$; NS; group × SD interaction: $F(2,30) = 0.93$; NS), correct responses latency (SD effect: $F(2,30) = 0.20$; NS; group effect: $F(2,30) = 2.14$; NS; group × SD interaction: $F(2,30) = 0.38$; NS), or latency to collect earned food pellets (SD effect: $F(2,30) = 0.54$; NS; group effect: $F(2,30) = 1.36$; NS; group × SD interaction: $F(2,30) = 0.96$; NS) (Table S6). Viral eGFP transduction did not affect performance, as the genotype effect with respect to omissions was still apparent after eGFP virus injection (KOeGFP vs. WTeGFP; $p < 0.05$).

Targeted re-expression of the β2-nACR subunit in the PrL area of the mPFC restores performance of the mice during a variable inter-trial interval procedure

We next compared performance of the injected mice in a variable inter-trial

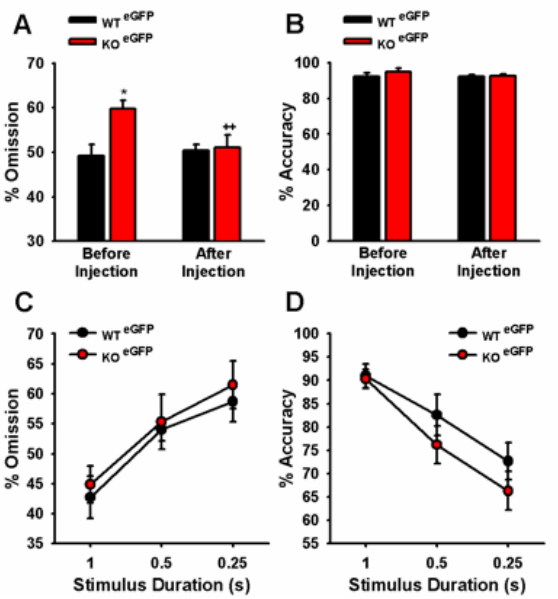

Figure S9. Targeted re-expression of the β2-nACR subunit in the PrL area of the mPFC restores performance of the mice during a variable stimulus procedure.
(A,B) Percentage of omission (A) and accuracy (B) in the 5-SCRTT during a variable stimulus procedure, in which stimulus durations were decreased (1, 0.5 and 0.25 s) for WTeGFP (black), KOeGFP (white) and KOVEC mice (red). * $p < 0.05$ and + $p < 0.05$, significant difference compared with WTeGFP and KOVEC, respectively, as revealed by the Newman-Keuls post hoc test. Data are shown as mean ± SEM.

interval (ITI) procedure, in which ITI durations were randomly increased to 7.5 and 12.5 seconds thus making the stimulus unpredictable. Under this task, there was an overall increase in percentage of omissions ($F(2,60) = 3.41$; $p < 0.05$) (Fig S10A). Re-expression of the β2 subunit in the PrL (KOVEC) was sufficient to restore the performance of the β2-/- mice on omissions, as they reached similar performance as WTeGFP mice ($F(2,30) = 3.60$; $p < 0.05$; WTeGFP vs. KOVEC; NS) and made less omissions than the KOeGFP mice (KOVEC vs. KOeGFP; $p < 0.05$). β2 re-expression in the PrL had no significant effect on accuracy (group effect: $F(2,30) = 015$; NS; ITI effect: $F(2,60) = 1.59$; NS; group × ITI interaction: $F(4,60) = 1.34$; NS) (Fig S10B).

Corroboration of the rescue-effect in two independent experiments

The effects of the β2 subunit re-expression in the mPFC were assessed in a second experiment (13 WTeGFP and 8 KOVEC mice). Under baseline training (SD1) (Fig S11A,B), the percentage of omissions showed a significant effect of lentiviral injection ($F(1,21) = 5.22$; $p < 0.05$), as well as a significant group × injection time interaction ($F(1,21) = 8.44$; $p < 0.01$) (Fig S11A). Post-hoc analysis revealed that β2 subunit re-expression in the mPFC of the KOVEC group significantly decreased the percentage of omissions (KOVEC before vs. KOVEC after, $p < 0.01$) to a level similar to WTeGFP mice (WTeGFP vs. KOVEC, NS). In contrast, β2 re-expression had no significant effect on accuracy (injection effect: $F(1,21) = 1.46$; NS; group × injection interaction: $F(1,21) = 1.62$; NS) (Fig S11B). Under greater attentional demand (Fig S11C,D), analysis performed on omission showed a significant effect of stimulus duration ($F(2,42) = 9.18$; $p < 0.001$), but no significant effect of group ($F(1,21) = 0.21$; NS) and no significant group × SD interaction ($F(2,42) = 0.55$; NS), indicating that KOVEC reached similar performance as WTeGFP mice (Fig S11C). β2 re-expression had no significant effect on accuracy (group effect: $F(1,21) = 1.78$; NS; SD effect: $F(2,42) = 11.08$; $p < 0.001$; group × SD interaction: $F(2,42) = 0.30$; NS) (Fig S11D).

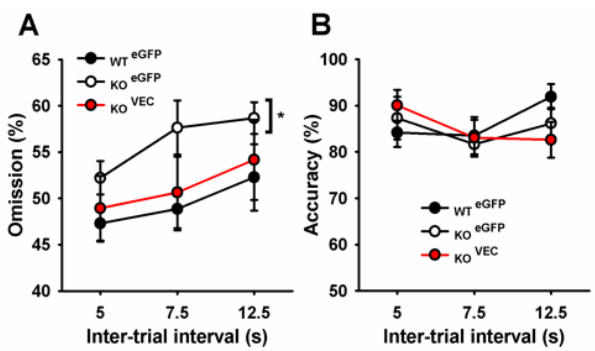

Figure S10. Targeted re-expression of the β2-nACR subunit in the PrL area of the mPFC restores performance of the mice during a variable inter-trial interval procedure.
(A,B) Percentage of omission (A) and accuracy (B) in the 5-SCRTT during a variable inter-trial interval procedure, ITI durations were increased (5, 7.5 and 12.5 seconds) for WTeGFP (black), KOeGFP (white) and KOVEC mice (red). * $p < 0.05$, significant difference compared with WTeGFP, as revealed by the Newman-Keuls post hoc test. Data are shown as mean ± SEM.

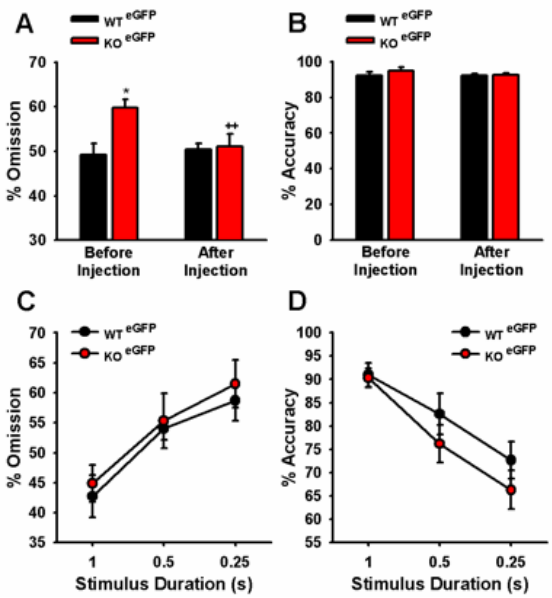

Figure S11. Corroboration of the rescue-effect in two independent experiments. (A,B) Percentage of omission (A) and accuracy (B) during baseline training (SD1) before and after viral injection for WTeGFP (n = 13, black) and KOVEC mice (n = 10, red). * $p < 0.05$, significant difference compared with WTeGFP; ++ $p < 0.01$, significant difference between before and after virus injection.
(C,D) Percentage of omission (C) and accuracy (D) in the 5-SCRTT during a variable stimulus procedure, in which stimulus durations were decreased (1, 0.5 and 0.25 s) for WTeGFP (black) and KOVEC mice (red). Data are presented as mean ± S.E.M.

Table S1: Performance of β2 $^{-/-}$ under baseline training condition of 1 s stimulus duration

Genotype	Number of trials	Omission (%)	Accuracy (%)	Premature	Correct latency (s)	Reinforcer latency (s)
WT	60.0 ± 0.0	47.3 ± 2.5	89.7 ± 1.4	1.45 ± 0.27	0.80 ± 0.03	2.00 ± 0.15
β2 $^{-/-}$	59.95 ± 0.05	57.1 ± 1.1 **	91.0 ± 1.5	1.43 ± 0.39	0.89 ± 0.05	2.09 ± 0.25

Data are presented as mean ± SEM. ** $p < 0.01$, significant difference between WT and β2 $^{-/-}$ mice.

Table S2: Performance of β2 $^{-/-}$ on the 5-CSRTT at varying stimulus durations

	Number of trials		Omission (%) ***		Accuracy (%) ***		Premature		Correct latency (s) *		Reinforcer latency (s)	
	WT	β2 $^{-/-}$	WT	β2 $^{-/-}$ ***	WT	β2 $^{-/-}$	WT	β2 $^{-/-}$	WT	β2 $^{-/-}$	WT	β2 $^{-/-}$
1.0	19.9 ± 0.1	20 ± 0.0	41.6 ± 1.7	52.0 ± 3.9 *	85.7 ± 2.7	93.8 ± 1.8	0.67 ± 0.27	0.71 ± 0.24	0.81 ± 0.06	0.76 ± 0.04	2.69 ± 0.5	2.59 ± 0.5
0.5	19.9 ± 0.1	20 ± 0.0	48.6 ± 2.4	59.5 ± 4.3 **	82.6 ± 4.4	85.2 ± 5.1	0.33 ± 0.28	0.35 ± 0.19	0.65 ± 0.05	0.63 ± 0.05	1.77 ± 0.3	2.15 ± 0.8
0.25	19.9 ± 0.1	20 ± 0.0	54.7 ± 2.5	68.0 ± 3.0 **	72.7 ± 3.9	69.1 ± 4.9	0.67 ± 0.29	0.41 ± 0.22	0.85 ± 0.10	0.71 ± 0.09	2.10 ± 0.2	2.38 ± 0.5

Data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, significant difference between WT and β2 $^{-/-}$ mice. * $p < 0.05$ and *** $p < 0.001$ for an overall effect of stimulus duration.

Table S3: Performance of α7 $^{-/-}$ under baseline training condition of 1 s stimulus duration

Genotype	Number of trials	Omission (%)	Accuracy (%)	Premature	Correct latency (s)	Reinforcer latency (s)
WT	58.9 ± 0.7	47.3 ± 3.2	84.4 ± 3.3	4.47 ± 1.27	0.74 ± 0.03	2.05 ± 0.12
α7 $^{-/-}$	59.6 ± 0.3	47.9 ± 2.5	84.4 ± 2.0	3.48 ± 2.99	0.81 ± 0.11	2.04 ± 0.35

Data are presented as mean ± SEM.

Table S4: Performance of α7 $^{-/-}$ on the 5-CSRTT at varying stimulus durations

	Number of trials		Omission (%) ***		Accuracy (%) ***		Premature		Correct latency (s)		Reinforcer latency (s)	
	WT	α7 $^{-/-}$	WT	α7 $^{-/-}$	WT	α7 $^{-/-}$	WT	α7 $^{-/-}$	WT	α7 $^{-/-}$	WT	α7 $^{-/-}$
1.0	19.9 ± 0.1	19.9 ± 0.1	37.8 ± 4.1	41.1 ± 2.3	86.1 ± 4.9	88.7 ± 2.6	0.50 ± 0.26	0.72 ± 0.22	0.71 ± 0.06	0.78 ± 0.03	2.01 ± 0.34	1.77 ± 0.11
0.5	20.0 ± 0.0	19.9 ± 0.1	53.8 ± 4.5	56.2 ± 3.3	79.2 ± 3.6	76.3 ± 4.1	0.58 ± 0.23	0.72 ± 0.21	0.57 ± 0.06	0.74 ± 0.07	1.86 ± 0.22	2.64 ± 0.59
0.25	20.0 ± 0.0	19.9 ± 0.1	57.5 ± 5.1	61.7 ± 3.4	73.3 ± 5.7	64.3 ± 4.1	0.67 ± 0.28	0.80 ± 0.22	0.65 ± 0.13	0.71 ± 0.07	1.65 ± 0.15	2.38 ± 0.38

Data are presented as mean ± SEM. *** $p < 0.001$ for an overall effect of stimulus duration.

Table S5: Performance of β2 $^{-/-}$ on the 5-CSRTT at 1 s stimulus duration before and after virus injection

	Number of trials		Omission (%) ***		Accuracy (%)		Premature		Correct latency (s)		Reinforcer latency (s)	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
WT^{eGFP}	59.1 ± 0.5	60.0 ± 0.0	43.6 ± 4.3	45.7 ± 1.9	84.6 ± 2.8	90.2 ± 2.1	1.43 ± 0.3	1.43 ± 0.3	0.77 ± 0.03	0.73 ± 0.03	1.72 ± 0.06	1.68 ± 0.09
KO^{eGFP}	59.3 ± 0.4	60.0 ± 0.0	59.4 ± 2.0 **	58.7 ± 1.6 **	90.1 ± 2.3	94.1 ± 2.1	1.62 ± 0.5	1.53 ± 0.4	0.81 ± 0.05	0.75 ± 0.06	1.76 ± 0.06	1.61 ± 0.08
KO^{VEC}	58.3 ± 0.7	59.3 ± 0.4	59.8 ± 1.8 **	47.5 ± 2.8 $^{+++}_{\$}$	88.3 ± 2.9	86.0 ± 2.2	1.48 ± 0.4	1.50 ± 0.4	0.78 ± 0.07	0.79 ± 0.04	1.76 ± 0.10	1.67 ± 0.08

Data are presented as mean ± SEM. ** $p < 0.01$, significant difference compared to WT^{eGFP}. $ $p < 0.05$, significant difference compared to KO^{eGFP}. +++ $p < 0.001$, significant difference between before and after virus injection.

Table S6: Performance of β2 $^{-/-}$ on the 5-CSRTT at varying stimulus durations after virus injection

	Number of trials			Omission (%) ***			Accuracy (%) ***			Premature			Correct latency (s)			Reinforcer latency (s)		
	WT^{eGFP}	KO^{eGFP}	KO^{VEC}	WT^{eGFP}	KO^{eGFP}	KO^{VEC}	WT^{eGFP}	KO^{eGFP}	KO^{VEC}	WT^{eGFP}	KO^{eGFP}	KO^{VEC}	WT^{eGFP}	KO^{eGFP}	KO^{eGFP}	WT^{eGFP}	KO^{eGFP}	KO^{VEC}
1.0	20 ± 0	20 ± 0	20 ± 0	42.7 ± 3.5	50.3 ± 2.2	44.5 ± 2.7	92.3 ± 2.9	93.8 ± 1.8	93.8 ± 1.8	0.8 ± 0.3	0.9 ± 0.3	0.8 ± 0.4	0.7 ± 0.02	0.7 ± 0.09	0.7 ± 0.03	1.5 ± 0.1	1.8 ± 0.2	1.3 ± 0.6
0.5	20 ± 0	20 ± 0	20 ± 0	49.1 ± 4.5	59.5 ± 3.7	53.2 ± 3.8	82.6 ± 4.4	85.2 ± 5.1	93.8 ± 1.8	0.4 ± 0.2	0.7 ± 0.3	0.3 ± 0.1	0.6 ± 0.03	0.6 ± 0.04	0.6 ± 0.07	1.6 ± 0.3	1.6 ± 0.3	1.9 ± 0.2
0.25	20 ± 0	20 ± 0	20 ± 0	54.1 ± 4.3	62.7 ± 3.3	57.3 ± 3.1	72.7 ± 3.9	69.1 ± 4.9	93.8 ± 1.8	0.3 ± 0.2	0.8 ± 0.3	1.0 ± 0.3	0.7 ± 0.07	0.6 ± 0.06	0.7 ± 0.05	1.6 ± 0.2	2.0 ± 0.3	1.9 ± 0.4

Data are presented as mean ± SEM. * $p < 0.05$ and $^{+}$ $p < 0.05$, significant difference compared to WT^{eGFP} and KO^{VEC}, respectively. +++ $p < 0.001$ for an overall effect of stimulus duration for an overall effect of stimulus duration.

Layer-specific modulation of the prefrontal cortex by nicotinic acetylcholine receptors.

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3 Chapter

Published in Cerebral Cortex 2013 Jan;23(1):148-161

Abstract

Acetylcholine signalling through nicotinic receptors (nAChRs) in the prefrontal cortex (PFC) is crucial for attention. Nicotinic AChRs are expressed on glutamatergic inputs to layer V (LV) cells and on LV interneurons and LVI pyramidal neurons. Whether PFC layers are activated by nAChRs to a similar extent or whether there is layer specific activation is not known. Here we investigate nAChR modulation of all PFC layers and find marked layer specificity for pyramidal neurons: LII-III pyramidal neurons and glutamatergic inputs to these cells do not contain nAChRs, LV and LVI pyramidal neurons are modulated by a7 and β2* nAChRs respectively. Interneurons across layers contain mixed combinations of nAChRs. We then tested the hypothesis that nAChRs activate the PFC in a layer specific manner using 2-photon population imaging. In all layers, nAChR-induced neuronal firing was dominated by β2* nAChRs. In LII-III only interneurons were activated. In LV and LVI both interneurons and pyramidal neurons were activated, the latter most strongly in LVI. Together these results suggest that in the PFC nAChR activation results in inhibition of LII-III pyramidal neurons. In LV and LVI nAChR-induced activation of inhibitory and excitatory neurons results in a net augmentation of output neuron activity.

Introduction

The prefrontal cortex (PFC) plays a central role in attention (Dalley et al., 2004; Groenewegen and Uylings, 2000). Acetylcholine critically modulates the PFC during attention behaviour (Dalley et al., 2004b; Parikh et al., 2007; Passetti et al., 2000) and shows rapid, phasic dynamics, on a seconds timescale (Parikh et al., 2007; Sarter et al., 2009). Nicotinic acetylcholine receptors (nAChRs), a subset of cholinergic receptors, are fast ionotropic receptors and their activation kinetics suggests that they are efficiently activated by these rapid increases in acetylcholine. Supporting this, mice lacking specific subunits of the nAChR show a decrement in attention performance (Bailey et al., 2010; Young et al., 2007, Guillem et al. 2011) and re-expression of the β2* subunit in the PFC improves attention of β2 null mice (Guillem et. al 2011). In addition, nicotinic receptor agonists acting on the PFC increase performance on these tasks (Hahn et al., 2003; Howe et al., 2010). To understand the role of nAChRs in cognitive functioning it is crucial to determine how nAChRs alter cortical information processing at the cellular and network level. Here we investigate what the relative impact of nAChR stimulation is on activity in different layers of the PFC network.

Neuronal network activation will strongly depend on which cell types express nAChRs as well as the subunit composition of the receptor. Pyramidal neurons in layer VI of the prefrontal cortex contain β2* nAChR accompanied by the accessory α5 subunit and these receptors activate output neurons that project to the medial dorsal thalamus (Kassam et al., 2008). Layer V pyramidal neurons are excited by nAChRs that enhance glutamatergic inputs through stimulation of presynaptic β2* nAChRs. nAChR modulation of glutamatergic inputs was abolished by lesioning the medial dorsal thalamus, showing that excitatory inputs from the medial dorsal thalamus to the PFC are specifically augmented by β2* nAChRs (Lambe et al., 2003). nAChRs also increase inhibition to layer V pyramidal neurons (Couey et al., 2007). In layer V regular-spiking non-pyramidal cells and low-threshold spiking cells express α7 and β2* nAChRs, whereas fast-spiking interneurons do not. In addition, low-threshold and fast-spiking interneurons are stimulated through presynaptic nAChRs on glutamatergic inputs (Couey et al., 2007; Poorthuis et al., 2009). What type of synaptic inputs and neurons in layer II-III are regulated by nAChRs is not known. It is also unknown whether layer VI interneurons are modulated by nAChRs. In this study, we address these issues. In addition, since nAChRs are found on both inhibitory and excitatory neurons in different PFC layers, it is not straight forward to predict how action potential firing of PFC output neurons is altered by nAChR stimulation. The nAChR distribution in the prefrontal cortex suggests a layer specific activation of the PFC by nAChRs. To test this hypothesis, we used two-photon calcium imaging of large scale PFC neuronal networks with single-cell resolution to assess how nAChR-induced activity is distributed across different layers. We find that nAChR-induced neuronal activity increases with depth in the cortex and is markedly different across

PFC layers due to specific distribution of β2* nAChRs.

Materials and methods

Prefrontal cortical slice preparation.

Prefrontal coronal cortical slices (300μM) were prepared from P14-P21 C57 BL/6 mice, in accordance with institutional and Dutch license procedures. Following rapid decapitation, the brain was removed from the skull in ice-cold artificial cerebrospinal fluid containing 125 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 3 mM MgSO4, 1 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose (~300 mOsm). After removal of the cerebellum the brain was glued on this plane to create a coronal orientation for cutting slices. Slices were then transferred into holding chambers containing aCSF 125 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 1 mM MgSO4, 2 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose (~300 mOsm) and bubbled with carbogen gas (95% O2 / 5% CO2) to recover for at least an hour.

Electrophysiology.

Slices were transferred to the recording chamber and perfused with standard aCSF (2-3 ml/min). All experiments were performed at 31-34° C. Cells were visualized using differential interfence contrast microscopy. Recordings were made using Multiclamp 700B amplifiers (Axon Instruments, CA), sampled at a frequency of 20 kHz, digitized by the pClamp software (Axon), and later analyzed off-line. Patch pipettes (3-5 MOhms) were pulled from standard-wall borosilicate capillaries and were filled with intracellular solution: 140mM K-gluconate, 1mM KCl, 10mM HEPES, 4mM K-phosphocreatine, 4 mM ATP-Mg, and 0.4 mM GTP (pH 7.2–7.3, pH adjusted to 7.3 with KOH) (290–300 mOsm) and biocytin (4mg/ml) (used for EPSC and puff application experiments, reversal potential chloride ~-127 mV, hence IPSCs in this case are detected as outward currents). Action potential profiles of cells were made using hyperpolarizing and depolarizing current steps. For IPSC experiments a modified intracellular solution was used with a high chloride concentration (70mM K-gluconate and 70 mM KCl) to augment GABAergic currents (reversal potential for chloride is ~-16 mV, hence GABA currents are detected as inward currents). All IPSC experiments were done in the presence of DNQX (10μM).

Nicotinic receptor currents on interneurons and pyramidal neurons were tested by pressure ejection of acetylcholine (Sigma, 1mM) for 100 ms using a Picospritzer III (General valve corporation, Fairfield, NJ) from a glass electrode with a tip opening of ~ 1 μm. The puffer pipette was located ~ 20 μm from the soma and placed along the axis of the apical dendrite either before or behind the soma. The presence of atropine (200 nM) prevented stimulation of muscarinic receptors and during all experiments DNQX (10 μM) and bicuculline (1 μM) were used to block synaptic transmission. For network experiments acetylcholine was bath applied.

Analysis and statistics for electrophysiological experiments.

Frequency and amplitude of PSC's were analyzed using MiniAnalysis (Synaptosoft, Inc). Local pressure application experiments were analysed using custom made software for Matlab (Mathworks). To test for frequency differences in PSC's we used a Student's t-test. To test for amplitude differences in PSC's we used a Kolmogorov-Smirnov test. To test for effects of pharmacology or genotype effects on nAChR currents induced by puff application of ACh a Student's t-test was used. To test for differences in ratio's of nAChR positive and negative cells in different layers we used a Chi-square test. Significant results were obtained with a p-value <0.05. p-values between 0.05 and 0.01 are shown as <0.05. p-values between 0.01 and 0.001 are shown as p<0.01 and p-values lower then 0.001 are shown as p<0.001.

Two photon calcium imaging.

Loading

Slices were made as described before, but in an alternative slicing solution (27mM NaHCO3, 1.5mM NaH2PO4, 222mM sucrose, 2.6mM KCl, 0.5mM CaCl2, 3mM MgSO4). Hereafter, slices were incubated in regular ACSF at 35°C for 20min and in room temperature for another 40min. For bulk loading, a modified protocol based on (Trevelyan et al., 2006) was used. Briefly, slices were first preincubated at 37°C for 5 min in 3ml ACSF containing 8µl Cremophor EL solution (0.5% Cremophor EL in DMSO). After this, 1µl Fura-2AM solution (25µg Fura-2AM with 4.5µl DMSO and 0.5µl pluronic acid) was pipetted on top of each slice. Then the slices were left for incubation for 35-40min after which they were put back in the slice chamber with ACSF at room temperature for at least 45min.

Imaging

Experiments were performed in ACSF (perfusion speed 2.5ml/min), continuously bubbled with 95% O2/ 5% CO2, at 32°C. Imaging was performed using a multibeam two-photon laser scanning microscope system (Trimscope, Lavision BioTec) coupled to a Ti:Sapphire laser (Chameleon, Coherent, excitation at 820nm) and a CCD camera (C9100 Hamamatsu). The objective used had a 20X magnification and a 0.95 numerical aperture. The imaged plane was always in the same orientation with respect to the pia and the distance between them was determined for later analysis. The imaged area was 300X300 µm (pixel size of 0.6µm, binning 2X2) and the imaging frequency was 9Hz.

Experimental protocol

Imaging was done during a 4 minutes baseline period, 2 minutes of ACh application and a period of 8 minutes while washing out the applied drugs.

Analysis

Analysis was done using custom made software for Matlab (Mathworks). This program detected cell contours, extracted the fluorescence within these contours as a function of time, and detected events, after which manual inspection was done in a blind fashion. Cells were divided in three depth groups, corresponding to the measured thicknesses of the three layers in the PFC. Neurons that were between 100 and 300 μm, between 300 and 550 μm and between 550 and 800 μm were considered to be part of respectively layer II/III, V and VI.

For determining the activity in the different drug conditions, the percentage of neurons showing at least one calcium event was calculated per slice per minute. If slices included multiple layers, then the slice was split up into two new slices containing just one layer. Effects of drugs, layer and condition were tested using repeated measures ANOVA which were, if significant, followed by Newman-Keuls posthoc tests.

After this, for direct comparison of the activations in the different conditions, it was determined per neuron whether the activity after ACh application was higher, lower or equal to the amount of calcium events in the minute before ACh application. Chi square tests were performed to test if this statistic was different for the multiple layers, condition and neuron types. In addition, binomial tests were used to determine the significance of the activation for every combination.

Determination of cell identity

High resolution z-stacks were made to optimize the possibilities for identification (voxel size: 0.4 X 0.4 X 0.5 μm). For the majority of neurons, proximal dendrites showed strong fluorescence.

Cells were only taken into account if dendritic fluorescence was sufficient and cells could be identified as interneurons or pyramidal neurons according to the following criteria: 1. the presence of a clear apical dendrite, 2. a pyramidal shaped cell body for pyramidal neurons. 3. a clear non-pyramidal cell body morphology. 4. bipolar or multipolar dendrite morphologies for the interneurons. Criteria 1 and 2 classified the neuron as pyramidal. Criteria 3 and 4 classified a neuron as interneuron. If the dendrites were not visible in the z-stack, the neurons were not categorized.

The identification of cells was done in a blind manner, i.e. the experimenter was unaware of whether neurons were activated by nicotine receptor stimulation or not, excluding the possibility of a bias. After morphological identification, data were compared to electrophysiological experiments.

If neurons could not be unequivocally identified, they were ecluded from statistics on cell type specific activation

Nissl staining

For Nissl staining five mice (P14-P19) were perfused with NaCl, followed by 4% PFA. After overnight fixation (4% PFA), slices of 100 μM were cut in sodium-acetate

using a vibratome. Slices were then photographed, to determine their size and to correct for shrinkage due to Nissl staining. Slices were then washed in sodium-acetate buffer (4x, 10 min) and made permeable by sodium-acetate + 0.5% TritonX (2 hours). As a final step they were were stained with 0.5% cresylviolet (10-15 minutes). Layer borders were determined by cytoarchitectonic criteria (Van De Werd et al.). Shown are the average layer depths of 9 slices along the rostral-caudal axis for all the animals.

Results

nAChR modulation of PFC pyramidal neurons

Pyramidal neurons in layer VI of the prefrontal cortex are modulated by α5-containing β2* nAChRs (Kassam et al., 2008). Layer V pyramidal neurons showed no response to local application of high doses of nicotine (10 μM) (Couey et al., 2007). Whether layer II-III pyramidal neurons in the prefrontal cortex are modulated by nAChRs is not known. Therefore, we tested whether PFC pyramidal neurons show inward currents upon direct ACh application. We made whole-cell recordings from PFC pyramidal neurons in the different layers (Figure 1) and used wildtype, β2-null or α7-null mice as well as pharmacological tools to determine the nAChR subunits involved. Pyramidal neurons were identified by their morphological appearance and based on the spiking profile in response to step depolarizations (Figure 1A1). During recordings neurons were filled with biocytin for post-hoc identification by cell morphology (Figure 1A1, 2A1 and 2C1). All recordings were done in the presence of 200 nM atropine to block muscarinic receptors, as well as DNQX (10 μM) and bicuculline (1 μM) to block synaptic transmission. Local pressure application of ACh (1mM, 100ms) onto the soma of LII-III pyramidal cells showed that only a very small fraction of cells (3 out of 24 neurons, 12.5 %) displayed an inward current (Figure 1A2-4; amplitude 29.6 ± 8.0 pA). These findings show that only a minority of layer II/III pyramidal neurons are modulated by nAChRs. The inward currents showed the rapid activation and desensitization kinetics characteristic of α7 nAChRs (McGehee and Role, 1995).

Next, we targeted layer V pyramidal neurons. In contrast to application of 10 μM nicotine (Couey et al., 2007), the majority of layer V pyramidal cells (27/31, 87 %) displayed a rapid inward current upon ACh application (73.4 ± 13.0 pA, n=27, Figure 1A2-4). The currents were reversibly blocked by the α7 nAChRs antagonist methyllycaconitine (MLA, 71.0 ± 14.8 pA vs -0.8 ± 0.9 pA [n=7], $p<0.01$). In addition, PFC layer V pyramidal neurons of transgenic mice lacking the α7 subunit showed no fast inward current (-0.4 ± 0.6 pA [n=8], $p<0.001$). These data suggest that the majority of Layer V pyramidal neurons contain functional α7 nAChRs.

In line with previous reports (Kassam et al., 2008), we found that layer VI pyramidal neurons all showed slow inward currents (61.0 ± 9.6 pA, n=17, Figure 1A2-4). The slow ACh-activated inward current activated was blocked by the antagonist

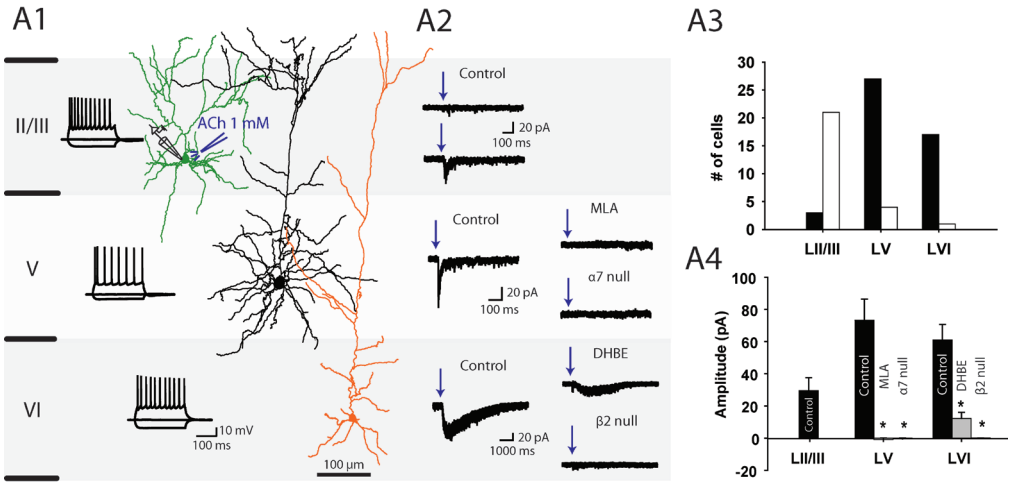


Figure 1. nAChR modulation of pyramidal neurons

(A1) Membrane potential responses to step current injections of pyramidal cells in the different layers of the medial PFC (current injections of -100 and +140 pA) and examples of post-hoc reconstructed pyramidal neuron morphologies.

(A2) Current responses of pyramidal neurons to local ACh (1mM) application (all experiments are in the presence of 200 nM atropine). Pyramidal neurons display a layer specific modulation by nAChRs.

(A3) Histogram quantifying the amount of pyramidal neurons positive (black) or negative (white) for functional nAChRs in each layer

(A4) Summary plot of average amplitudes of the ACh-induced currents. Currents in layer V pyramidal neurons were blocked by the α7 antagonist MLA (n=7, Student's t-test, $p<0.01$) and absent in mice lacking the gene for the α7 subunit (n=8, Student's t-test, $p<0.001$). Currents in layer VI pyramidal neurons were blocked by β2* antagonist DHBE (n=8, Student's t-test, $p<0.01$) and absent in β2-null mice (n=7, Student's t-test, $p<0.001$).

of β2-containing nAChRs dihydro-β-erythroidine (DHβE, 74.5 ± 18.5 pA vs 12.3 ± 3.8 pA [n=7], $p<0.01$) and was absent in transgenic mice lacking β2 subunits (3.0 ± 0.7 pA [n=7], $p<0.001$). However, in contrast to earlier reports, we did find that a subset of layer VI pyramidal cells showed an additional α7-like current (5/25, 20%, Supplemental Figure 2). Taken together, these data show that a distinct modulation of pyramidal neurons by nAChRs exists in different prefrontal cortical layers. The majority of pyramidal neurons in layer II/III do not contain nAChRs. Pyramidal neurons in layer V are modulated by nAChRs containing α7 subunits, whereas pyramidal neurons in layer VI are under regulation of nAChRs containing β2 subunits which are occasionally accompanied by α7-like currents (summarized in figure 8).

nAChR modulation of excitatory synaptic transmission in PFC layers

In addition to direct depolarization by postsynaptic nAChRs on pyramidal neurons, nAChRs located on presynaptic glutamatergic inputs can augment the activity of pyramidal neurons by increasing glutamatergic signalling (McGehee et al., 1995; Mansvelder and McGehee, 2000). Excitatory inputs to PFC Layer V neurons

were strongly augmented by activation of nAChRs containing β2 subunits (n=4, Supplemental Figure 2, (Couey et al., 2007; Lambe et al., 2003). This augmentation was blocked by TTX (92.7 ± 18.9 % of control, n=4, p <0.01, Supplemental Figure 2), indicating that nAChRs are located on axonal compartments away from the presynaptic terminal. We investigated whether similar mechanisms exist in pyramidal neurons in other cortical layers by making simultaneous recordings of multiple pyramidal neurons in different layers and monitoring spontaneous excitatory transmission (Figure 2), which was sensitive to DNQX (10 μM, Supplemental Figure 2). In stark contrast to the frequency increase of excitatory postsynaptic currents (EPSCs) found in all layer V pyramidal neurons (991.6 ± 172 % of control, n=21, p<0.01, Figure 2A3, 2C3 and 2E1), the frequency of EPSCs received by the simultaneously recorded layer II-III pyramidal neurons showed no change (112.8 ± 8.4 %, n=8, p=0.08, Figure 2A2-3, 2D-E). The amplitude distribution of the EPSCs in layer II/III pyramidal neurons did not change in the presence of ACh (n=8, p>0.05 for all cells, Figure 2A4) also in contrast to the increase in amplitude by ACh application found in all layer V pyramidal neurons (n=20/21, p<0.05, data not shown). Similar to ACh, application of nicotine (10μM) did not alter the frequency and amplitude of EPSCs in layer II/III pyramidal neurons (106.7 ± 4.2 %, n=10, p=0.34, Figure 2B1). To test whether a potential effect of nAChR activation on excitatory transmission could be masked by a simultaneous effect of ACh on inhibition (Couey et al., 2007), bicuculline (10 μM) was applied to block inhibitory transmission. No effect was found of nAChR stimulation on the frequency of EPSCs received by layer II/III pyramidal neurons (127.3 ± 9.5 %, n=4, p=0.06, Figure 2B3). These findings show that ACh does not modify excitatory synaptic transmission received by layer II/III pyramidal neurons, which suggests that glutamatergic inputs to these neurons do not contain functional nAChRs.

Simultaneous recordings of layer V and VI pyramidal neurons showed that layer VI pyramidal neurons experienced a significantly smaller increase in EPSC frequency by ACh application than layer V pyramidal neurons (p<0.01). Only a transient small increase was detected (185.1 ± 32.0 %, n=8, p=0.03, Figure 2C2, 2C3, 2D-E). An increase in amplitude was detected in only one out of eight cells (Figure 2C4). These data show that excitatory inputs to layer VI pyramidal neurons are mildly modulated by ACh through nAChR activation. In conclusion, in contrast to layer II-III pyramidal neurons, both layer V and layer VI pyramidal neurons experience increased frequencies of excitatory inputs in the presence of ACh (summarized in figure 8).

Modulation of inhibitory inputs to pyramidal neurons by nAChRs is limited to layers II/III and V.

Besides pyramidal neurons, also interneurons are modulated by nAChRs. In layer V, nAChRs are expressed by different types of interneurons (Couey et al., 2007). Activation of nAChRs on interneurons increases action potential firing and

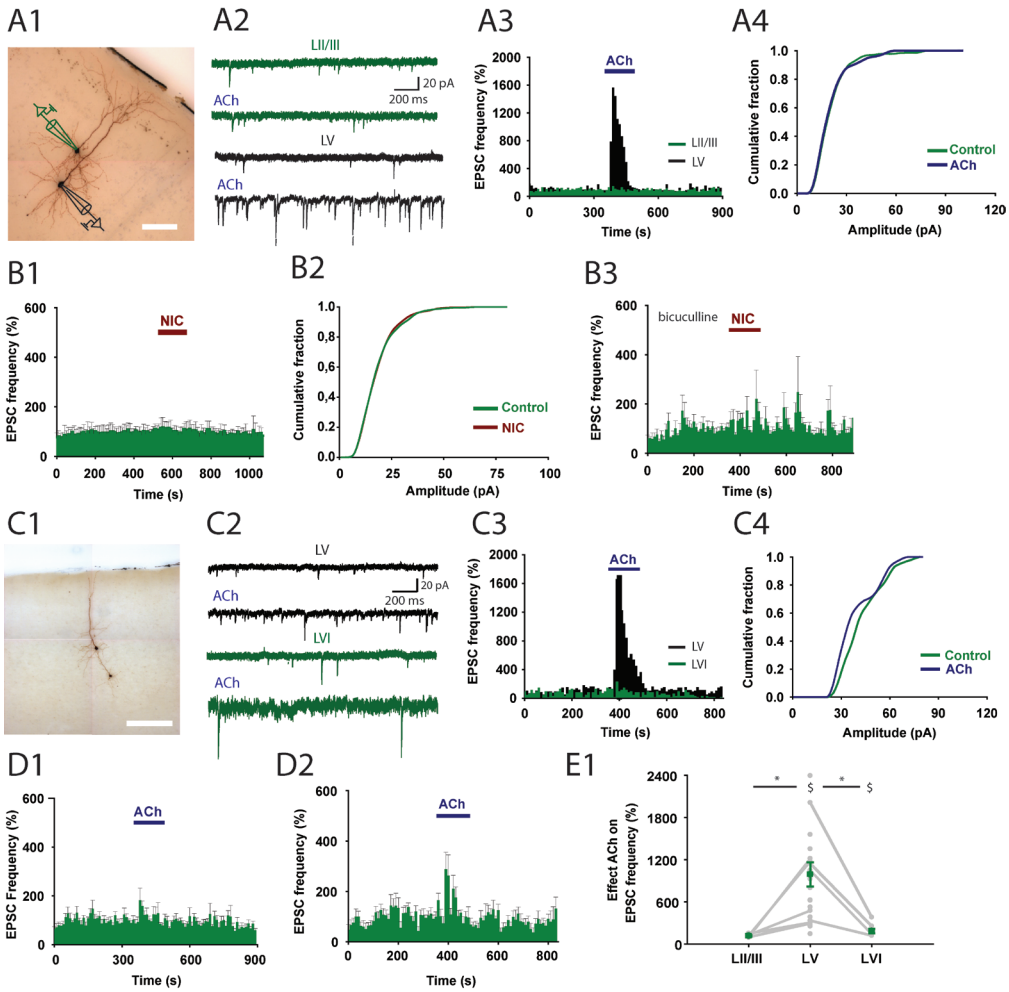


Figure 2. nAChR modulation of glutamatergic synaptic transmission

(A1) Biocytin-filled cells showing the morphology of two pyramidal neurons in layer II-III and V recorded simultaneously. On top the recording setup for data in (A2-4) is depicted. Scale bar = 100 µm.

(A2) Example traces of spontaneous excitatory transmission (EPSC's) during baseline (top) and ACh (1mM) application (bottom) for a double recording of LII-III (green) and LV (black).

(A3) Histogram of the EPSC frequency. Same neurons as in B2.

(A4) Cumulative amplitude distribution of EPSC's recorded from a layer II-III pyramidal neuron. ACh application had no effect on the distribution (Kolmogorov-Smirnov test, $p>0.05$)

(B1) Histogram showing the average EPSC frequency over time during nicotine (10µM) application (n=11)

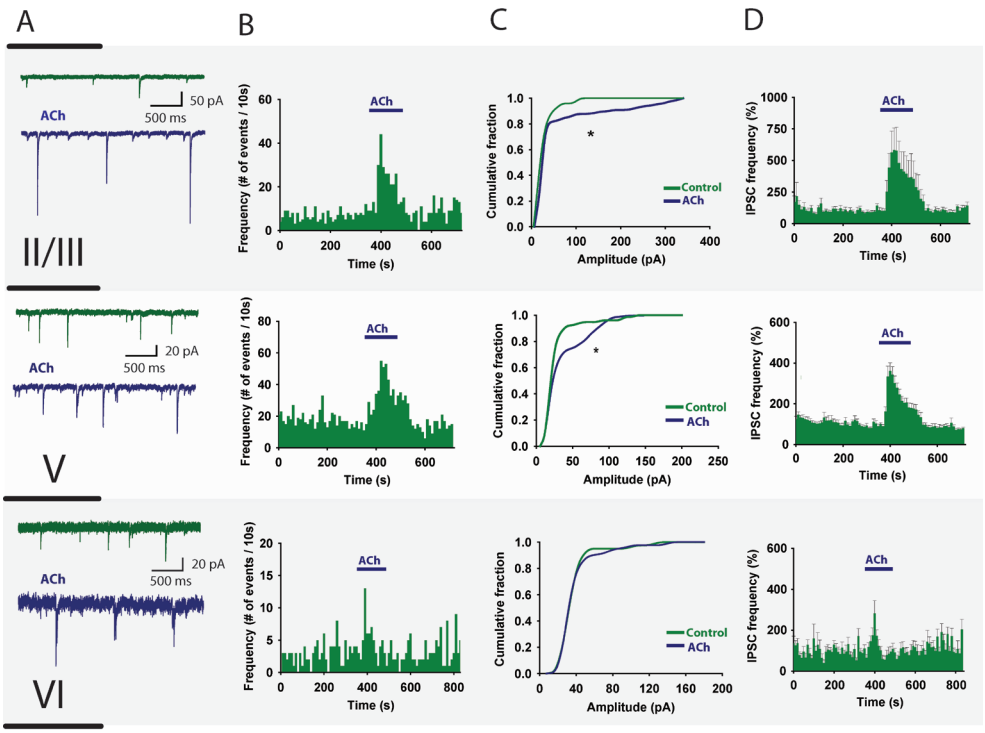
(B2) Cumulative amplitude distribution of EPSC's recorded from a layer II-III pyramidal neuron. Nicotine (10µM) application had no effect on the distribution (n=11, Kolmogorov-Smirnov test, $p>0.05$)

(B3) Same experiment as in B1 in addition of the GABAa receptor blocker bicuculline (10µM)

(C1-3) Same as (A1-3) but now for a layer V (black) and LVI pyramidal neuron (green). Scale bar = 250 µm.

(C4) Cumulative amplitude distribution of EPSC's recorded from a layer VI pyramidal neuron. ACh application had no effect on the distribution (Kolmogorov-Smirnov test, $p>0.05$).
(D1) Average EPSC frequency histogram of layer II-III pyramidal neurons (n=8). Duration of ACh application is indicated by blue bar.
(D2) Average EPSC frequency histogram of layer VI pyramidal neurons (n=8). Duration of ACh application is indicated by blue bar.
(E1) Summary plot of ACh effects on EPSC frequency in pyramidal neurons from different layers. Individual recordings are shown with grey dots. Simultaneous recordings in different layers are connected with grey line (n=6 layer II-III and LV, n=4 layer V and LVI). Green dot shows average frequency during acetylcholine application. Layer V and VI showed a significant increase in EPSC frequency on ACh application (n=21, Student's t-test, $p<0.01$ and n=8, Student's t-test, $p=0.03$). The increase in EPSC frequency was significantly lower in LVI (Student's t-test, $p<0.01$). All error bars in figure indicate SEM.

augments the frequency of GABAergic inputs to pyramidal neurons (Alkondon et al., 2000; Couey et al., 2007; Ji and Dani, 2000). It is not known whether interneurons in other medial PFC layers contain functional nAChRs. It is also not known whether pyramidal neurons in layer II/III and VI of the prefrontal cortex experience increased inhibition when nAChRs are activated. We hypothesized that interneurons in cortical layers II-III and VI are also modulated by nAChRs. To test this, we made simultaneous whole cell recordings of pyramidal neurons in different layers and monitored inhibitory postsynaptic currents (IPSCs) upon ACh application (Figure 3A). All currents could be blocked by the GABAa receptor antagonist bicuculline (10 μM, n=5, Supplemental Figure S3). In layer II/III pyramidal neurons, application of ACh increased the frequency of IPSCs ($p<0.05$ for 8 out of 10 neurons; Figure 3A-B and 3D). In simultaneously recorded layer V pyramidal neurons a similar increase in IPSC frequency upon ACh application was found in 15 out of 16 cells ($p<0.05$, Figure 3A-B and 3D). Between layer II/III and layer V pyramidal neurons, no significant difference in frequency increase was observed ($p=0.5$, Figure 3D). In both layers, the amplitude of IPSCs showed a shift towards larger amplitudes in the majority of pyramidal cells (6 of 10 layer II-III neurons, 10 of 16 layer V neurons, $p<0.05$, Figure 3C). In both layer V and layer II/III pyramidal neurons the effects on frequency and amplitude of IPSCs were blocked by the antagonist for nAChRs mecamylamine (LII-III: 505.3 ± 148.2 % of control [n=10] vs 117.9 ± 9.2 % [n=3], $p<0.05$. LV: 351.2 ± 42.8 % [n=16] vs 87.4 ± 3.4 % [n=3], $p<0.01$), Figure 3E and Supplemental Figure 2) and sodium channel blocker TTX (LII-III: 505.3 ± 148.2 % [n=10] vs 101.3 ± 13.8 % [n=2], $p<0.05$. LV: 351.2 ± 42.8 % [n=16] vs 108.6 ± 8.2 % [n=3], $p<0.01$, Figure 3E and Supplemental Figure 2). This suggests that these nAChR effects are not mediated by presynaptic receptors, but by receptors located on perisomatic compartments of interneurons. In contrast, the majority of layer VI pyramidal neurons showed no changes in IPSC frequency or amplitude in response to ACh application (10 of 12 cells showed no response, $p>0.05$, Figure 3A-D). In 2 of 12 pyramidal neurons ACh did increase IPSC frequency and amplitude (not shown). Taken together, these data suggest that in layers II/III and V inhibitory synaptic transmission received by pyramidal neurons



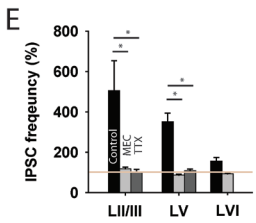


Figure 3. nAChR modulation of inhibitory transmission received by pyramidal neurons

(A) Example traces showing spontaneous IPSCs recorded from PFC pyramidal neurons in the absence (green) and presence of 1mM ACh (blue).

(B) Histograms showing the frequency of IPSC's during a single experiment.

(C) Cumulative amplitude distribution showing that during acetycholine application IPSC's with larger amplitude appeared in layer II-III (6 out of 10 neurons) and LV (10 out of 16 neurons, Kolmogorov-smirnov test, $p < 0.05$), but not in layer VI (10 out of 12 neurons, Kolmogorov-smirnov test, $p > 0.05$).

(D) Average IPSC frequency histogram for pyramidal neurons in different PFC layers during acetylcholine application. Blue bar indicates the time when acetylcholine is present. Acetylcholine significantly increased the IPSC frequency in LII-III (505.3 ± 148.2 %, Student's t-test, $p<0.01$) and LV (351.2 ± 42.8 %, $p<0.001$), but not in LVI (153.6 ± 18.4 %, $p=0.09$).

(E) Summary bar graph quantifying the effect of acetylcholine and different blockers on the IPSC frequency measured in prefrontal cortical pyramidal neurons (Mecamylamine; LII-III: 505.3 ± 148.2 % of control [n=10] vs 117.9 ± 9.2 % [n=3], $p<0.05$. LV: 351.2 ± 42.8 % [n=16] vs 87.4 ± 3.4 % [n=3], $p<0.01$), TTX; (LII-III: 505.3 ± 148.2 % [n=10] vs 101.3 ± 13.8 % [n=2], $p<0.05$. LV: 351.2 ± 42.8 % [n=16] vs 108.6 ± 8.2 % [n=3], $p<0.01$).

is augmented by nAChR stimulation, whereas in layer VI inhibitory inputs to the majority of pyramidal neurons are not controlled by nAChRs.

nAChR modulation of PFC non-fast-spiking interneurons

Different types of interneurons in PFC layer V express various types of nAChRs (Couey et al., 2007). Which interneurons in layer II/III and VI contain nAChRs is not known. Using wildtype, β2-null and α7-null mice as well as pharmacology in whole-cell recordings from interneurons, we tested which interneurons subtypes are modulated by nAChRs. We distinguished between two types of inhibitory neurons: fast-spiking interneurons and non-fast-spiking interneurons (Kawaguchi and Kubota, 1997), which can be distinguished based on morphology and action potential firing characteristics (Figure 4A1, A2, Figure 5A1, A2 and Supplemental Table 1). Fast-spiking interneurons (FS) showed high action potential firing frequency and had a low input resistance (183.0 MΩ ± 13) and narrow spike width (0.49 ms ± 0.02, n=32, Supplemental Table 1 and Figure 5). Non-fast-spiking cells (NFS) showed a broader spike width (0.96 ms ± 0.03, $p<0.05$) and a higher input resistance (331 MΩ ± 13, $p<0.05$, n=76, Supplemental Table 1 and Figure 4). Upon direct application of ACh (in the presence of atropine, 200 nM, and DNQX, 10 μM, and bicuculline, 1μM) NFS cells in layers II/III and V showed mixed responses (Figure 4A3). In layer II/III, 29 of 47 cells showed a response to ACh (Figure 4B1). In 9 of these 29 NFS neurons a slow current was activated that was most likely mediated solely by nAChRs containing the β2 subunit (Figure 4A3-5, B1, B2), since slow currents were blocked by DHβE and absent in β2-null mice (Figure 4A5). 7 of 29 NFS cells showed only a rapid α7-like inward current.. Rapid inward currents that were present in β2-null mice were blocked by MLA (n=5, $p<0.01$, Supplemental Figure 2) and absent in α7-null mice (Figure 4A3-5, B1, B2). Close to half of the NFS cells in which ACh induced an inward current, a mixed current was detected that was most likely mediated by both β2 and α7 nAChRs (Figure 4A3, B1). MLA blocked the fast component of this current, whereas DHβE blocked the slow component (n=2, Figure 4A4). Within the group of non-fast-spiking neurons a subpopulation of neurons expresses somatostatin. These cells mainly target distal tufts of pyramidal cells (Kawaguchi and Kondo, 2002; Silberberg and Markram, 2007) and have been shown to express nAChRs in layer V (Couey et al., 2007). To test whether somatostatin positive cells in LII-III also contain functional nAChRs we used the GIN-line that expresses eGFP in somatostatin positive cells in superficial layers (Ma et al., 2006). All somatostatin positive cells tested showed inward currents upon ACh application. Most cells showed slow inward currents reminiscent of b2*-like nAChRs (4 out of 5 cells, 80%), whereas one cell displayed the rapid α7-like nAChR response (Figure 4B3).

In layer V, a larger proportion the NFS cells showed an inward current upon direct ACh application (85.7 % vs 68.4 %, $p<0.001$, Figure 4B1). ACh induced in 18 of 21 layer V NFS cells mixed inward currents (Figure 4A3). As with layer II/III NFS cells, these fell into three groups: one group of NFS cells showed only β2-like nAChR mediated slow currents (Figure 4A3). A second group showed only fast a7-like inward currents, whereas the third group showed a mix of fast and slow currents (Figure 4A3,

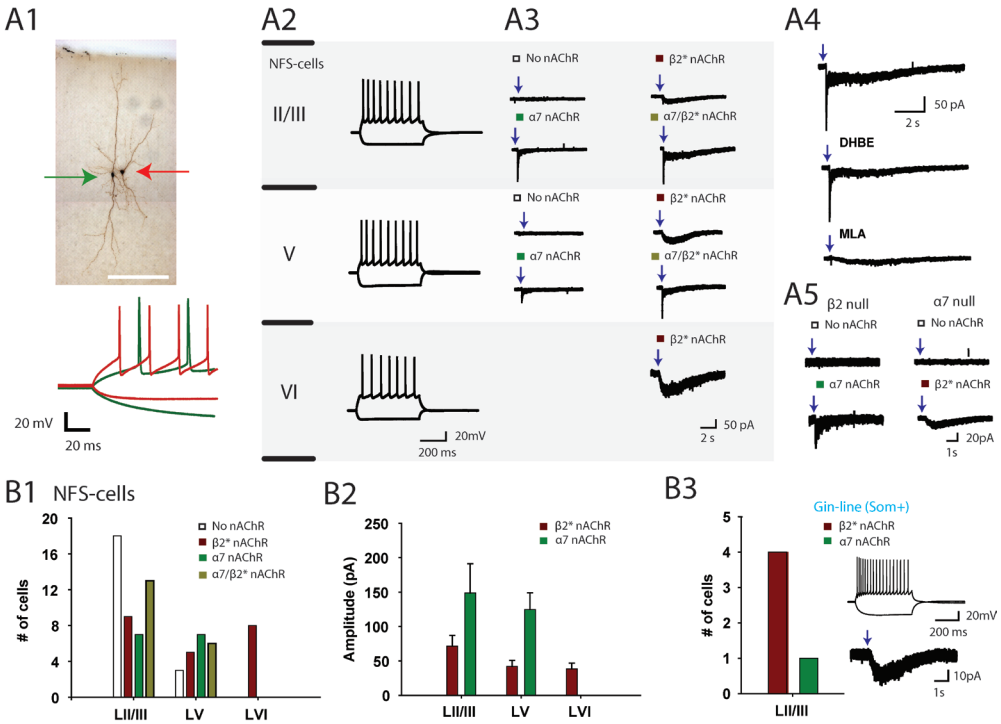


Figure 4. nAChR modulation of non-fast-spiking interneurons
(A1) Biocytin staining of a NFS- and FS-interneuron in layer V. Lower panel shows characteristic action potential firing of a NFS- and FS-cell. Scale bar is 250 µM
(A2) Spike profile of NFS interneurons in different layers (Current injection -100 and +140 pA)
(A3) Example traces of ACh-induced responses in NFS interneurons. Fast α7-like, slow β2*-like and mixed nAChR responses were observed.
(A4) NFS cell showing mixed nAChR reponse. Fast α7-like components were blocked by MLA (10nM), whereas the slow β2*-like component was blocked by DHBE (10µM).
(A5) Example traces of NFS cells recorded in β2 and α7-null mice. β2-null mice only exhibited fast onset nAChR responses or cells without a nAChR response. α7-null mice showed either no nAChR response or slow nAChR responses.
(B1) Histogram summarizing the nicotinic receptor distribution on NFS cells for each layer
(B2) Summary of the α7-like and β2*-like nAChR-mediated responses in different layers
(B3) Somatostatin-positive cells expressing eGFP are positive for β2*-like nAChR-mediated currents.

B1, B2).

In layer VI, all 8 recorded NFS cells showed slow ACh-induced inward currents of 38.4 ± 8.4 pA (Figure 4A3, B1, B2). Thus, nAChR modulation of NFS interneurons in PFC is layer specific, with a higher proportion of NFS cells in deep layers that contain functional nAChRs. β2-containing and α7 nAChRs are found separately or on the same neuron in layers II/III and V. Layer VI NFS neurons display currents reminiscent of β2-containing nAChRs (summarized in figure 8).

nAChRs modulation of fast-spiking interneurons

In contrast to NFS cells, fast spiking cells in all PFC layers did not show β2-containing nAChR-mediated inward currents upon ACh application. All inward currents recorded in FS cells had fast onset and decay kinetics, reminiscent of α7 nAChRs, and were 78.3 ± 20.7 pA (LII-III) and 64.9 ± 11.2 pA (LV) in amplitude (Figure 5A3, B1, B2). Layer II-III contained the highest proportion of nAChR containing FS cells. 9 of 11 FS neurons showed the fast inward current that was blocked by MLA (n=3, p=0.04, Figure 5A3, B3), and was absent in α7 null mice (n=2, p<0.01, Figure 5B3). In layer V, 7 of 13 cells expressed fast ACh-induced inward currents (Figure 5B1, B2). In layer VI, six FS cells were detected and they did not contain nicotinic receptors. Taken together, the majority of FS cells in layers II/III and about half of the FS cells in layer V contain functional nAChRs of the α7 subtype (summarized in figure 8).

Network modulation of the prefrontal cortex by nAChRs

Cholinergic signalling in the PFC can occur on a timescale of seconds to minutes (Parikh 2007 en 2009). Our data show that nicotinic receptors are expressed by inhibitory neurons in layer II-III and by both excitatory and inhibitory neurons in layer V and layer VI. How activation of the nAChRs on these opposing types of neurons affects the balance of neuronal activity in the different layers when acetylcholine levels increase in the PFC is not known. In addition, layer V pyramidal neurons get activated through presynaptic β2* nAChRs, whereas layer VI pyramidal neurons are stimulated through postsynaptic β2* receptors directly. How activation of the receptors alters the balance of activity among these different layers is not known. Given the distribution of nAChRs across the layers, we hypothesize that activation of the PFC by nAChRs is layer specific. To test this, we monitored neuronal activation in different layers with single cell resolution by two-photon imaging of medial PFC slices that were bulk-loaded with the calcium indicator fura-2-AM (Figure 6). Changes in fluorescence were proportional to the amount of action potential firing in activated neurons, as reported in the literature (Supplemental Figure 1, (Cossart et al., 2005)). The depth of prefrontal cortical layers II-III, V and VI was determined by post hoc Nissl staining (Figure 6C-D). Subsequently, neurons were assigned to layers by their distance from the pia. After baseline activity was recorded in the presence of the muscarinic receptor blocker atropine (200nM) for at least 4 minutes, acetylcholine (ACh, 1mM) was applied for two minutes. During baseline, neuronal activity was low: every minute on average 2.46% ± 0.35% of the neurons (n = 82 slices) exhibited at least one fluorescence transient (Figure 6A-B and 6E). During wash in of ACh, a higher proportion of the cells in the slices displayed fluorescence transients (Figure 6A-B and 6E). Increased activity was found across all layers (Figure 6E). Neuronal activity in LVI (550-800 μm) was most strongly affected by nAChR stimulation (14.2 +/- 3.1 % active cells, p<0.001). In contrast, in LII-III (100-300 μm) only moderate numbers of

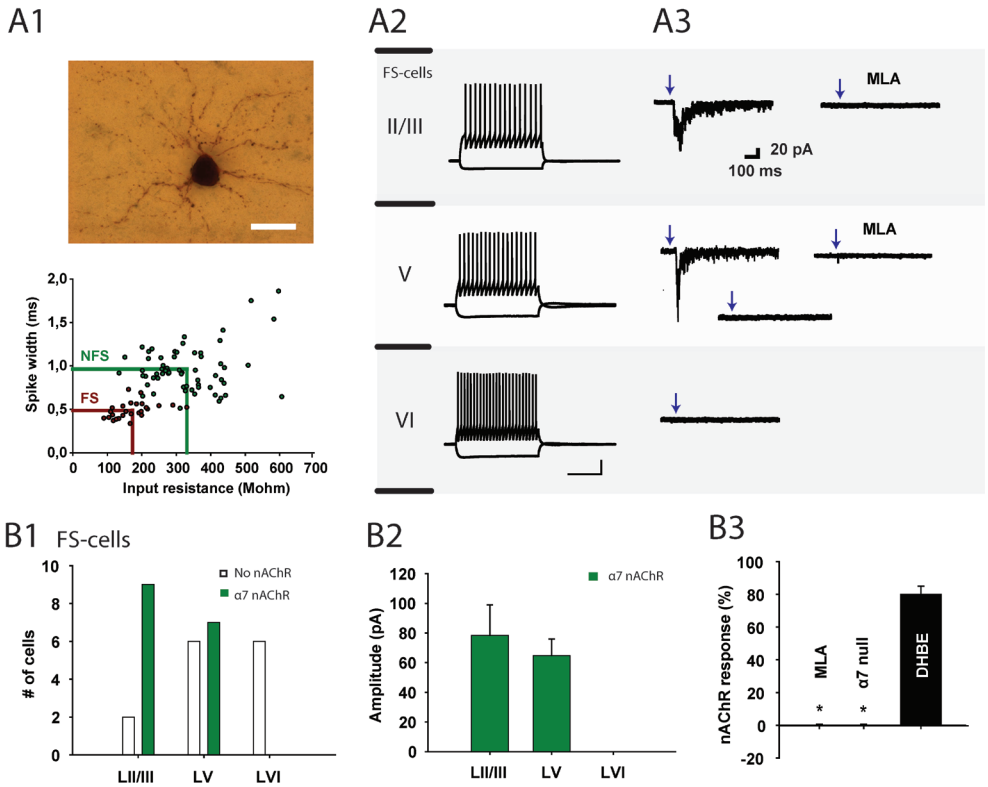
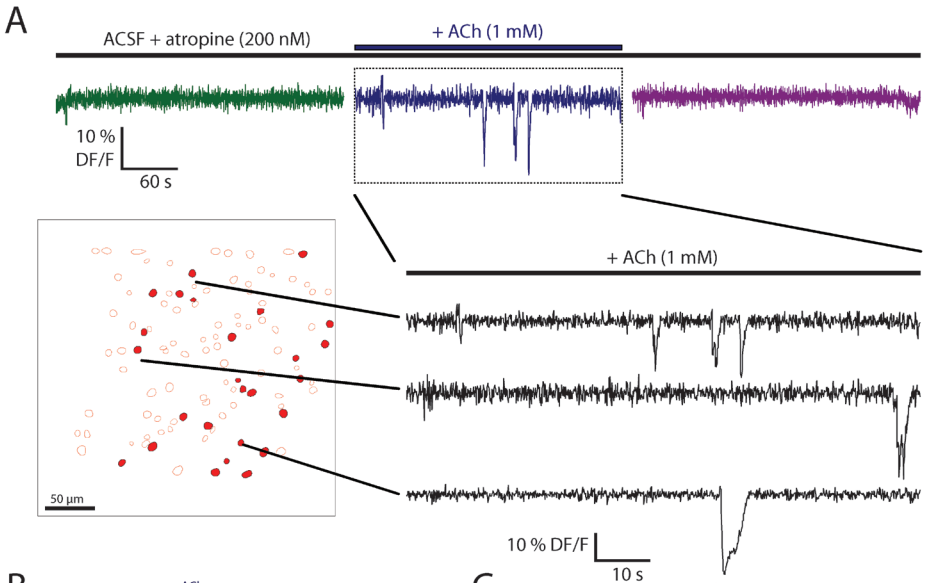
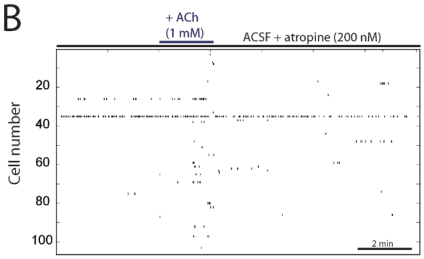


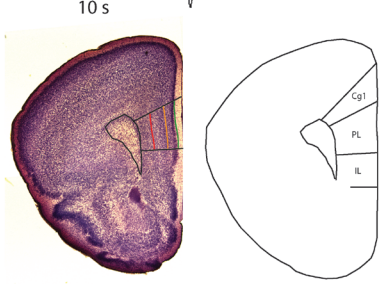
Figure 5. nAChR modulation of fast-spiking interneurons
(A1) Biocyting staining of a FS-interneuron. Lower panel indicates spike-width and input resistance for FS and NFS-cells. Scale bar = 25 µm.
(A2) Spike profile of FS interneurons in different layers
(A3) Example traces of ACh-induced responses in FS interneurons. α7-mediated responses were observed in layer II-III and half of the layer V neurons (positive and negative example shown), but not in layer VI.
(B1) Histogram summarizing the amount of FS cells positive for nAChRs.
(B2) Summary of the average amplitudes of α7 currents on FS cells per layer.
(B3) All nAChR induced currents on FS cells could be blocked by MLA (n=3), but not by DHBE (n=2). FS cells in α7-null mice were not found to express functional nAChRs (n=2).

neurons were activate during nAChR stimulation (5.7 +/- 1.8 %, p=0.24; Figure 6C). Nicotinic AChR activation induced intermediate amounts of activity in LV (300-550 µm; 9.6 +/- 1.3 %, p<0.01). Baseline activity was not significantly different between layers and wash out of ACh resulted in activity levels similar to baseline (p=0.87, 0.97 and 0.64 for respectively LII/III, V and VI). nAChR induced activity was completely absent in the presence of the nAChR antagonist mecamylamine (10µM, -0.3 +/- 1.6 % activation, p=0.85).

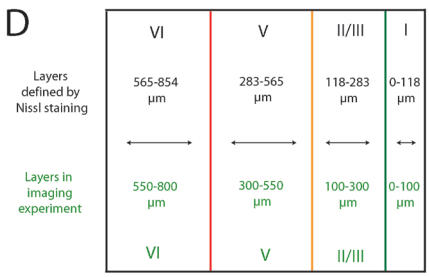
The cell types that were activated by nAChR stimulation were identified from

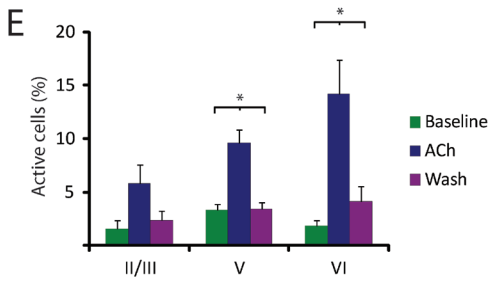


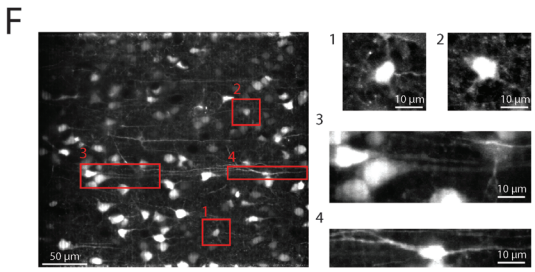




Prelimbic cortex







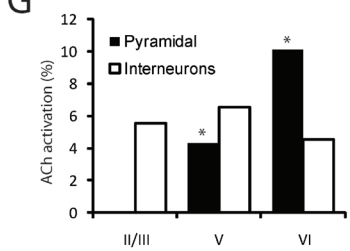


Figure 6. ACh-induced neuronal activation is most prominent in deep layers.
(A) Top traces: schematic representation of the experimental protocol and an example of the fluorescence of one cell during the entire experiment. Bottom panels: Example of automatically detected cell contours and fluorescence traces from these cells. Cells that are indicated in red showed increased activity during ACh application. Fluorescence traces of three high-lighted neurons during ACh application show downward deflections that indicate calcium influx due to neuronal activity.
(B) Rasterplot of network activity during a single experiment. Black ticks represent the occurrence of a calcium event. During application of ACh, there is an increase in the number of cells showing calcium events.
(C) Left: Example of a Nissl stained coronal slice, prelimbic area is indicated by black lines. Colored lines indicate the depth of layers within the prelimbic area (Yellow is LII-III, red is LV and LVI is black). Right: Schematic cartoon indicating the medial prefrontal cortical areas.
(D) Overview of the average depth of prefrontal cortical layers defined by Nissl staining (upper black values) and depth of layers used to categorize the imaged cells (lower green values).
(E) Percentage of cells that is active per minute in different layers (n=82 slices). ANOVA repeated measure testing indicated that the drug effect ($p<0.001$) and its interaction with the layers ($p<0.05$) are significant. In deep layers there is a significant effect of ACh application (layer V: $p<0.01$ (n=37); layer VI: $p<0.001$ (n=23, Newman-Keuls posthoc test)). During washout, activity returned to baseline (layer V: $p<0.01$; layer VI: $p<0.001$).
(F) Example of identification of neurons. On the left a collapsed z-stack at high resolution. On the right examples of identified neurons interneurons (1,2 & 4) and pyramidal neurons (3)
(G) Percentage of identified pyramidal and interneurons which activity was increased during ACh application. Pyramidal cells showed a significant increase in the percentage of cells that was active in both layer V ($p<0.01$) and layer VI ($p<0.001$, binomial test). In addition, the size of the activation was significantly different between layers ($p<0.01$). Interneuron activation was not significantly different between the three layers
All error bars represent SEM.

high resolution z-stacks of the imaged slices (See Methods). More than half of the individual neurons could be identified as either pyramidal or interneuron based on morphology (Figure 6F). We found that nAChR stimulation activated interneurons similarly across all layers (Figure 6G). Layer II-III pyramidal neurons showed no change in activity upon nAChR stimulation ($p=0.6$). This is in contrast to layer V and layer VI where, besides interneurons, also pyramidal neurons were prominently activated by nAChR stimulation ($p<0.01$ and $p<0.001$). The increase in pyramidal neuron activity was significantly different for the PFC layers ($p<0.01$) (Figure 6G). Taken together, nAChR stimulation results in more neuronal activity in LV and LVI of the PFC activating both pyramidal and interneurons. In superficial layers fewer cells show activity upon nAChR stimulation and these are all interneurons.

Neuronal network activation by nAChRs is mediated by β2 nAChRs*

Our results show that neurons in the PFC can be stimulated through both α7 and β2-containing nAChRs. Modulation of layer VI neurons is dominated by β2* nAChRs. However, LV pyramidal neurons are stimulated through presynaptic β2* nAChRs and postsynaptic α7 nAChRs. Also interneurons in LII-III and LV show mixed β2* and α7 responses. To investigate the contribution of α7 and β2* nAChR in inducing network

activity during ACh concentration changes at the scale of seconde to minutes, we imaged neuronal activity upon ACh bath application in β2 and α7 null mice (Figure 7). Both WT and α7 null mice show strong layer dependent activations (Figure 7A-C (WT: $p<0.001$; α7 null: $p<0.001$): WT layer II/III: $p<0.01$; layer V: $p<0.001$; layer VI: $p<0.001$; α7 null: layer II/III: ns; layer V: $p<0.001$; layer VI: $p<0.001$) whereas none of the layers showed a significant activation in β2 null mice (Figure 7A-C). The activation was significantly stronger in PFC slices of WT and α7-null than in β2-null animals in layer V and layer VI (WT vs β2 null: layer V: $p<0.001$; layer VI: $p<0.001$; α7 null vs β2 null: layer V: $p<0.001$; layer VI: $p<0.001$). Hence, although layer V neurons show prominent expression of α7 nAChRs on pyramidal and interneurons, no change in activity was found. Only in LII-III we did not find a significant activation upon application of ACh in α7 null mice, but activity levels were not statistically different from wildtype.

Thus, nAChR-induced neuronal activation across PFC layers strongly depends on β2-containing nAChRs when ACh levels rise in a sustained manner on a time scale of seconds to minutes. These data may suggest that the relatively slow changes in ACh concentrations do not result in strong enough inward currents mediated by α7-containing nAChRs to induce action potential firing by neurons. To test this, we recorded from layer V pyramidal neurons and compared the peak amplitude of α7 currents induced by puff application and bath application on the same cells. Peak amplitude was high during puff application, while bath application resulted in low amplitude currents (Supplemental figure 4). This is in contrast to β2* nAChRs on layer VI pyramidal neurons which reach similar peak amplitudes during both types of ACh application. These data suggest that when ACh levels rise in the PFC on the scale of seconds to minutes (Sarter 2009), neuronal activation is predominantly mediated by β2-containing nAChRs .

Discussion

Activation of the prefrontal cortex by nAChRs will depend on which cell types express nAChRs and what subunits they are made of. Since nAChRs modulate excitatory as well as inhibitory neurons in the PFC circuitry, an understanding of how the PFC output is affected by nAChR activation requires an integrated view of nAChR-induced activity in the PFC. Using a combined approach of whole-cell recordings and two-photon network imaging we find in this study that (1) PFC pyramidal neurons in different layers show a differential pattern of nAChR modulation: layer II/III pyramidal neurons do not contain nAChRs, layer V pyramidal neurons contain α7 nAChRs, and Layer VI pyramidal neurons are modulated by β2* receptors; (2) Glutamatergic inputs to layer II-III are not regulated by nAChRs in contrast to excitatory inputs to layer V and layer VI pyramidal neurons; (3) Interneurons show differential patterns of nAChR modulation, in layer II-III and V α7 and β2* nAChRs are found, whereas

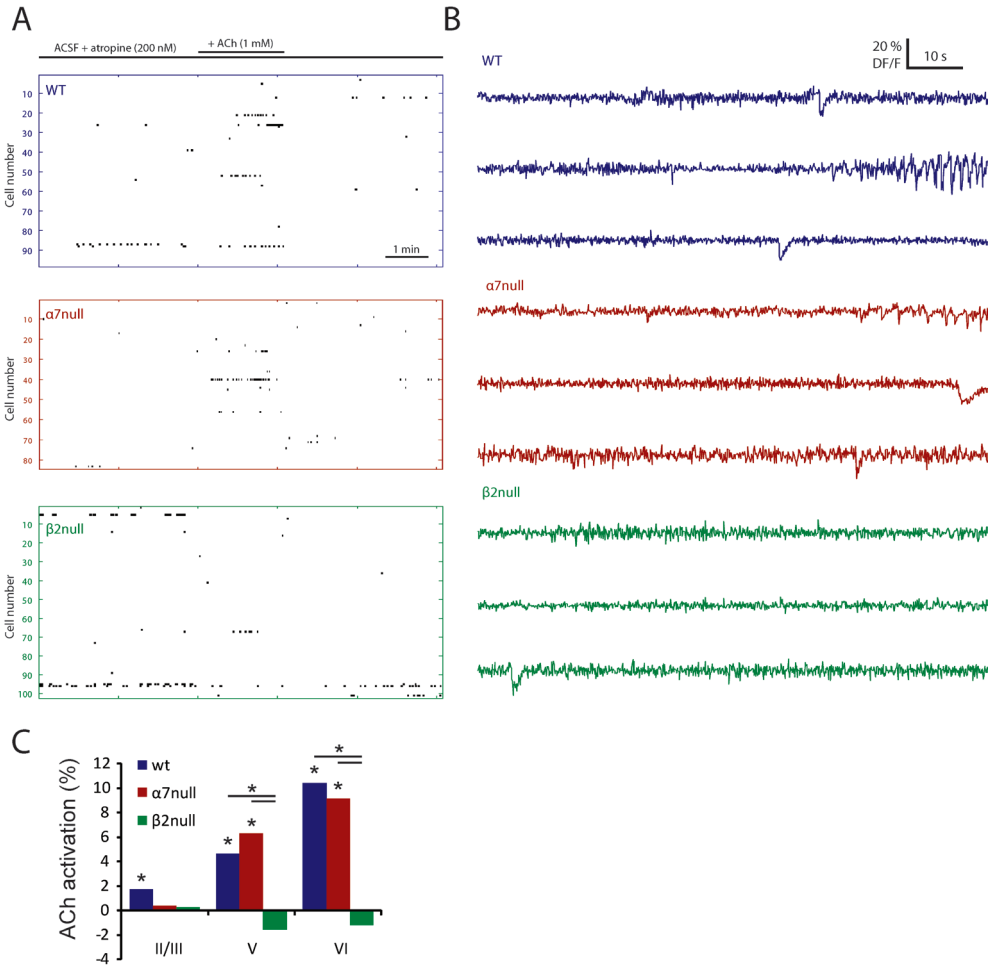


Figure 7. ACh induced network activity in α7 and β2 null mice
Activation of PFC neurons by ACh (1mM) in mice lacking β2 and α7 subunits show a strong contribution of β2 subunits to the observed activation of the network.
(A) Rasterplot of network activity in slices from wildtype, α7 null and β2 null mice.
(B) Fluorescence traces during ACh application for wildtype, α7 null and β2 null mice.
(C) Summary histogram showing that both WT and α7 null mice show strong layer dependent (WT: $p<0.001$; α7 null: $p<0.001$) activations (binomial tests: WT layer II/III: $p<0.01$; layer V: $p<0.001$; layer VI: $p<0.001$; α7 null: layer II/III: ns; layer V: $p<0.001$; layer VI: $p<0.001$) whereas none of the layers shows a significant activation in β2 null mice. The activation is significantly stronger in PFC slices of WT and α7-null than in β2-null animals in layer V and layer VI (WT vs β2 null: layer V: $p<0.001$; layer VI: $p<0.001$; α7 null vs β2 null: layer V: $p<0.001$; layer VI: $p<0.001$).

in layer VI only β2* nAChRs are found (Summarized in Figure 8); (4) nAChRs stimulate both excitatory and inhibitory neurons in layer V and VI and this results in a net augmentation of activity of layer V and VI pyramidal neurons, whereas in layer

II-III only interneurons are activated; (5) Network activity in the PFC in response to bath application of ACh is layer specific and dominated by β2* nAChRs.

Layer specific nAChR modulation of PFC pyramidal neurons.

In the PFC, nAChR expression is found across all layers (Gioanni et al., 1999). nAChRs can alter pyramidal neuron activity by enhancing glutamatergic inputs or by activating postsynaptic receptors directly (Poorthuis et al., 2009). Hippocampal pyramidal neurons express functional α7 nAChR (Ji et al., 2001). In motor cortex, somatosensory cortex and visual cortex, layer II-III and layer V pyramidal neurons do not contain nAChRs (Gil et al., 1997; Gulledge et al., 2007; Nicoll et al., 1996; Porter et al., 1999; Xiang et al., 1998). We find that PFC layer II-III pyramidal cells also do not contain nAChRs, and also glutamatergic inputs to these pyramidal neurons are not modulated by nAChRs. Hence, nAChRs do not augment the output of superficial pyramidal neurons.

In contrast, in layer V pyramidal neurons, activation of presynaptic β2* nAChRs on glutamatergic inputs from the thalamus strongly enhances activity of these neurons (Couey et al., 2007; Gioanni et al., 1999; Lambe et al., 2003). We find that these presynaptic mechanisms are specific to layer V as they are absent in layer II-III and VI. This may suggest that nAChR-mediated modulation of thalamic inputs to the PFC is specifically targeting layer V pyramidal neurons, which project to the striatum and hypothalamus (Gabbott et al., 2005). Nicotinic enhancement of thalamic inputs to the cortex also plays a role in primary sensory areas, where it enhances sensory representation in the cortical target structure (Disney et al., 2007; Kawai et al., 2007; Penschuck et al., 2002). In addition to presynaptic β2* nAChRs that can augment its activity, layer V pyramidal neurons also contain postsynaptic α7 nAChRs. In contrast to layer V, excitatory glutamatergic inputs to layer VI pyramidal neurons were mildly modulated by nAChRs. As was reported (Kassam et al., 2008), we found that these neurons are modulated by β2* nAChRs that are responsible for the strong activation of the layer VI neuronal population. Layer VI pyramidal neurons in entorhinal cortex also have been reported to be modulated by non-α7 nAChRs, most likely containing β2 subunits (Tu et al., 2009).

Modulation of PFC interneurons by nAChRs.

Interneurons form a highly diverse group of cells with distinct roles in cortical computation (Kawaguchi, 1993; Markram et al., 2004). Here we distinguished between fast-spiking and non-fast-spiking cells, as well as somatostatin positive cells, a subgroup of non-fast-spiking cells. Fast-spiking cells target the perisomatic region of pyramidal neurons (Kawaguchi and Kondo, 2002; Kawaguchi and Kubota, 1997) and are therefore thought to be involved in regulating the activity window of pyramidal neurons. In somatosensory areas fast-spiking cells regulate feedforward inhibition of incoming thalamic inputs (Sun et al., 2006). Feedforward inhibition in the PFC

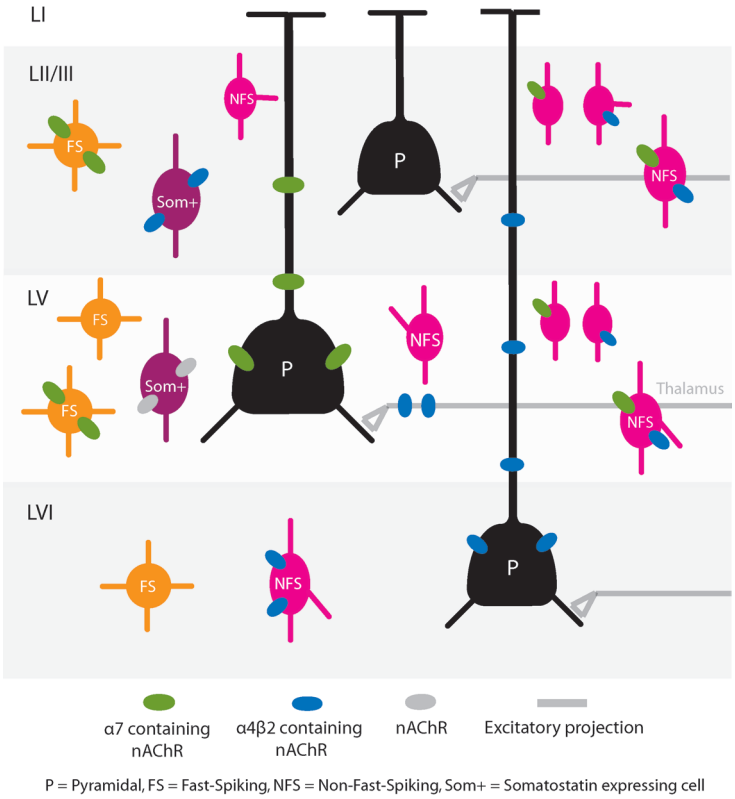


Figure 8. Overview of nicotinic receptor modulation of the different cell types in all PFC layers.

plays an important role in the integration of hippocampal inputs, which enter the PFC through superficial layers (Jay and Witter, 1991; Tierney et al., 2004). We find that fast-spiking cells in layer II-III contain α7 nAChRs, as do about half of the fast-spiking cells in layer V. This contrasts to studies that report the absence of nAChRs on these neurons, which might be attributable to the use of a different agonist (Couey et al., 2007) or species and age differences (Gulledge et al., 2007). nAChR activation on fast-spiking interneurons in PFC layer II/III may alter processing of hippocampal inputs.

Somatostatin-positive cells target distal dendritic regions (Kawaguchi and Kondo, 2002; Silberberg and Markram, 2007), and can mediate disynaptic inhibition between pyramidal neurons (Kapfer et al., 2007; Silberberg and Markram, 2007). We found that all cells that are regular-spiking and somatostatin-positive in layer II-II and V were positive for nAChRs, suggesting that nAChRs play an important role in modulating feedback inhibition among pyramidal neurons in these layers.

Increased inhibition through activation of nAChRs expressed by interneurons has been found in many different brain regions (Alkondon et al., 2000; Gulledge et al., 2007; Ji and Dani, 2000; Jones and Yakel, 1997; Mansvelder et al., 2002; McQuiston

and Madison, 1999; Xiang et al., 1998). When activated by nAChR stimulation, interneurons can alter activity and plasticity in pyramidal neurons (Alkondon et al., 2000; Couey et al., 2007; Ji and Dani, 2000; Ji et al., 2001; Xiang et al., 1998). Increased inhibition can lead to blockade of LTP induction in the hippocampus (Ji et al., 2001) and an increase in the threshold for induction of spike-timing dependent plasticity (Couey et al., 2007). Similar mechanisms may play a role across PFC layers since we find that non-fast-spiking cells in all layers express nAChRs.

An integrated view of PFC neuronal network modulation by nAChRs.

An understanding of how information processing in cortical networks is altered by nAChR activation requires an integrated view of nAChR activation across all layers, with cellular resolution. Using voltage-sensitive dye imaging, Tu et al. (Tu et al., 2009), found that in entorhinal cortex low concentrations of nicotine predominantly activate neuronal populations in layer VI. However, the identity of the activated neurons could not be confirmed during these experiments due to lack of cellular resolution. Two-photon imaging offers cellular resolution while simultaneously monitoring the activity of hundreds of neurons (Cossart et al., 2005). Using this method, we find that in the PFC distinct populations of neurons are activated by nAChR stimulation in a layer specific manner. As in entorhinal cortex (Tu et al., 2009), we find that in the PFC nAChR stimulation results in the strongest activation of layer VI neuronal populations. These populations consist of both pyramidal neurons and interneurons. A similar picture is seen in layer V, but β2* nAChRs located on presynaptic terminals activate layer V output neurons to a lesser extent then postsynaptic β2*nAChRs in layer VI pyramidal neurons. In contrast, nicotinic AChR-mediated activation of neuronal populations in layers II/III only consisted of interneurons. When monitoring network activity induced by ACh, we found that in all layers the amount of nAChR-induced neuronal activity in α7-null mice is comparable to that seen in wildtype mice. In contrast, in layer II-III and V networks of β2-null mice nAChR-induced neuronal activity is absent. Thus, during bath application of ACh, nicotinic receptor modulation of the activity in PFC layer II-III and V neuronal populations is dominated by β2* nAChRs. We show that α7 nAChRs are not efficiently activated by slow increases in acetylcholine as delivered through bath application. The amplitude of the depolarizing current reached by bath application is low and probably does not induce action potential firing in these cells. β2 receptors are efficiently activated by both types of ACh application. Part of cholinergic signalling in the PFC happens on the scale of seconds to minutes (Sarter 2009). Hence these data suggest that during prolonged high levels of acetylcholine, the increase in neuronal activity is mainly mediated by β2* nAChRs. For induction of neuronal action potential firing by α7 nAChR stimulation, faster sub-second cholinergic signals may be required.

Changing levels of nAChR modulation and expression over development.

nAChR expression levels change over development. For the PFC it has been shown that β2* nAChR responses to acetylcholine of LVI pyramidal neurons decrease with age in the prefrontal cortex (Kassam et al. 2008). Also α7 nAChRs are known to be highly expressed in developing networks and distribution and expression changes in the developing cortex but expression persists into adulthood (Tribollet et al. 2004), although this is not specifically known for the PFC. Nicotinic receptors might therefore be involved in the development of neuronal networks. Indeed, α7 nAChRs have indeed been implicated in the formation of thalamocortical synapses (Broide et al. 1996) and β2* nAChRs in the structural development of PFC LVI pyramidal neurons (Bailey et al. 2011). In this study we used young PFC slices. Nicotinic receptor modulation of the PFC might be altered in adult animals.

Functional implications.

Rapid acetylcholine release is critical for attention performance (Parikh et al., 2007). Nicotine can enhance attention performance by acting on nAChRs in the PFC (Hahn et al., 2003). Accumulating evidence indicates that β2* nAChRs have a central role in regulating neuronal networks involved in attention. First, Specific re-expression of the β2 subunit in β2 null mice increases attention performance (Guillem et al 2011). Second, diminishing endogenous activation of β2* receptors by deleting its accessory α5* subunit leads to decreased attention performance in the 5-choice serial reaction time task (Bailey et al., 2010). Thirdly, β2* nAChR agonist are more efficient in enhancing cognitive performance in attention tasks compared to nicotine that acts on both β2* and α7* nAChRs (Howe et al., 2010). We found that β2* nAChRs are the main receptor subtype altering activity within the neuronal network of the prefrontal cortex upon prolonged slow application of ACh. Layer II-III pyramidal neurons are inhibited by nAChR stimulation. Layer V and layer VI pyramidal neurons, which connect to subcortical output structures, get prominently activated by nAChR stimulation. Layer VI pyramidal neurons, which get most prominently activated, project mainly to the medial dorsal thalamus, whereas layer V neurons project mainly to the striatum and hypothalamus (Gabbott et al., 2005). How acetylcholine and nicotine alter prefrontal cortical network activity in different cortical layers during attentional tasks is not known. Our data shows that nAChRs regulate prefrontal cortical circuitry in a layer specific manner. Hence when studying the modulatory effects of acetylcholine release and nicotine on attention performance in vivo, layer specificity is a critical factor.

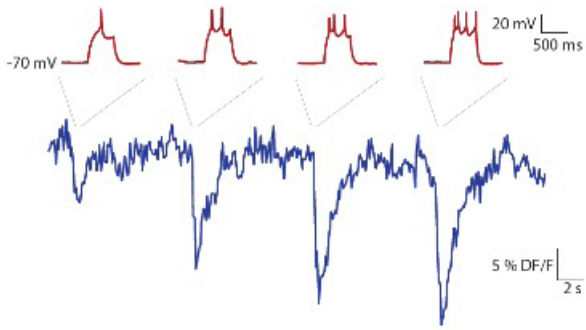
Acknowledgements

The authors wish to thank Prof. Guus Smit and dr. Rhiannon Meredith for valuable comments on the manuscript, Zimbo Boudewijns for exquisite assistance performing Nissl stainings, Prof. Uwe Maskos for sharing α7 and β2-null mice, Prof. Harry

Uylings for insightful discussions on analyzing Nissl stainings, Brendan Lodder for Neurolucida reconstructions, and Hans Lodder, Jaap Timmerman and Tim Heistek for excellent technical assistance. Funding for this work was provided by grants from NWO (917.76.360), VU University board, Neuroscience Campus Amsterdam (NCA) and the European Research Council (ERC) to HDM.

Supplementary material

Supplementary Figures (S1-S4).



Supplementary figure 1, related to figure 6. These data show that calcium events, as seen in our two-photon data in figure 1, are associated with action potential firing in recorded neurons. Simultaneous fluorescence imaging and intracellular stimulation showing the relationship between the number of action potentials and the size of the calcium transients. Action potentials were evoked every 15s by injecting increasing amounts of current. Action potentials displayed are truncated.

Nicotine (10μM) application had no effect on the distribution (n=11, Kolmogorov-Smirnov test, $p>0.05$)
(C3) Same experiment as in C1 in addition of the GABAa receptor blocker bicuculline (10μM)
(D1) Histogram showing the average EPSC frequency over time during acetylcholine (1mM) application for layer V pyramidal neurons (n=21)
(C2) Cumulative amplitude distribution of EPSC's recorded from a layer V pyramidal neuron. Acetylcholine (1mM) application shifted the amplitude distribution towards larger amplitudes (n=20/21, Kolmogorov-Smirnov test, $p<0.05$)
(E1) Histogram showing the average EPSC frequency during acetylcholine application for β2 wildtype mice (n=4)
(E2) Histogram showing the average EPSC frequency during acetylcholine application for β2-null mice (n=4)
(F1) Histogram showing the average EPSC frequency during acetylcholine application in the presence of TTX (1μM, n=4)
(F2) Cumulative amplitude distribution of EPSC's recorded from a layer V pyramidal neuron. Acetylcholine (1mM) application did not shift the amplitude distribution towards larger amplitudes in the presence of TTX (n=4, Kolmogorov-Smirnov test, $p>0.05$)

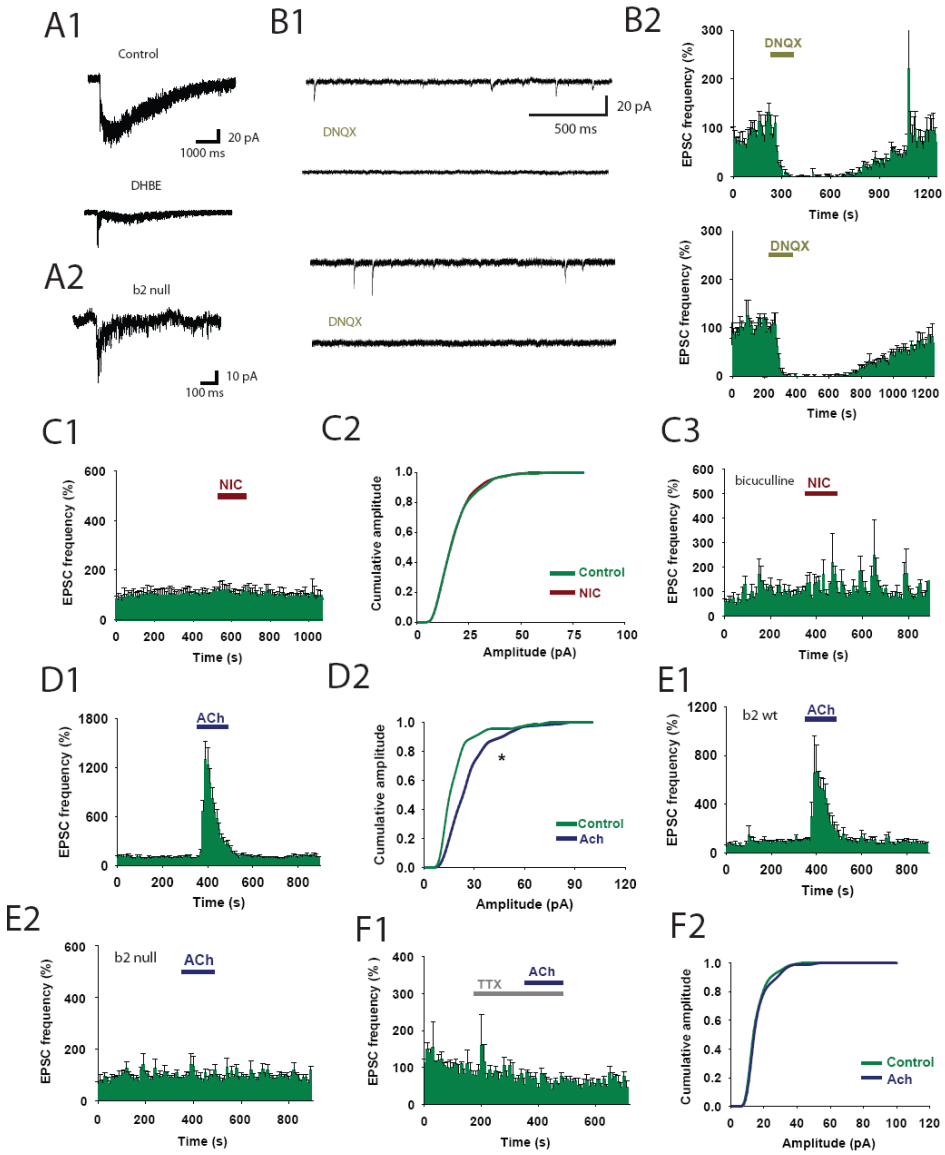


Figure S2, related to figure 2: Pharmacology excitatory nAChR modulation of pyramidal neurons.
(A1) Example recording of a LVI pyramidal neuron showing a mixed α7 and b2* response as revealed by blockade of the slow β2* component by DHBE.
(A2) Example recording of an α7 positive LVI pyramidal neuron in a β2-null mouse.
(B1) Example traces of dual EPSC recording during control conditions and application of the AMPA/Kainate receptor blocker DNQX (10μM).
(B2) Histogram showing the average EPSC frequency over time during DNQX application (n=4)
(C1) Histogram showing the average EPSC frequency over time during nicotine (10μM) application (n=11)
(C2) Cumulative amplitude distribution of EPSC's recorded from a layer II-III pyramidal neuron.

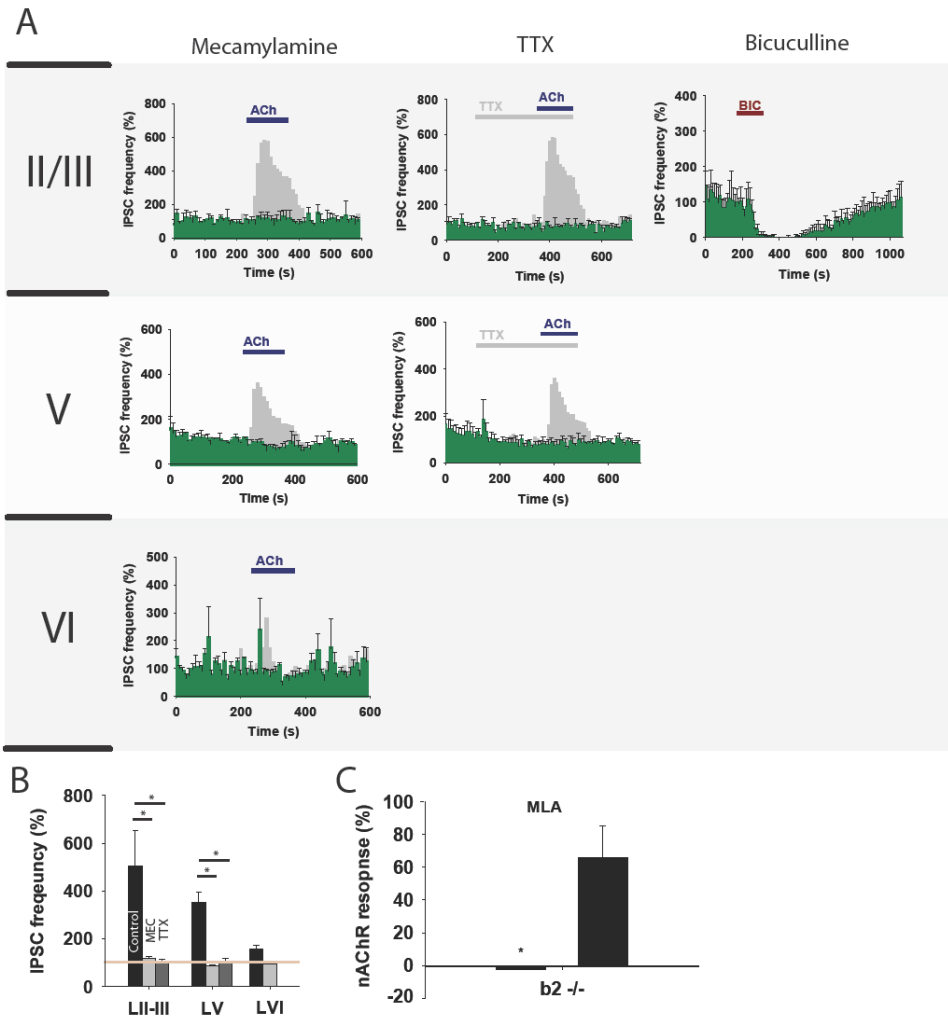


Figure S3, related to figure 3. Pharmacology nAChR modulation of inhibitory transmission to pyramidal neurons

(A) Average histograms showing the effect of acetylcholine in the presence of mecamylamine (1μM), tetrodotoxin (1μM) and bicuculline (10μM) for the different layers. In grey the control respons is shown.
(B) Summary bar graph quantifying the effect of acetylcholine and different bockers on the IPSC frequency measured in prefrontal cortical pyramidal neurons.
(C) Histogram showing the quantification of nAChR positive cells in β2 null mice. All currents are reversibly blocked by the α7* antagonist MLA (10nM).

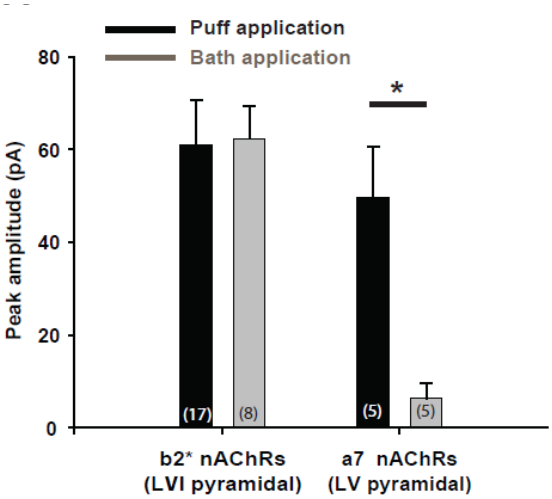


Figure S4, related to figure 7. α7 nAChRs are do not reach high peak amplitude when activated by bath application of 1 mM acetylcholine.
(A) Summary histogram showing the peak amplitude calculated for b2* nAChRs on LVI pyramidal neurons during fast (puff) and slow (bath) application of 1mM Ach (puff and bath application were done on separate cells). While β2* nAChRs reach similar peak currents during fast and slow application of ACh, α7 nAChRs do not reach high amplitude when activated by bath application of Ach (experiments done in the presence of DHβE). This is in contrast to the high amplitude seen in the same cells by puff application. These data might explain that in β2 null mice no activity is seen upon washin of acetylcholine.

FS

Layer	*n-number*	*Ir*	*Tm*	*Spike width*	*Spike threshold*
All	32	183.0 (13)*	9.5 (0.7)*	0.49 (0.02)*	-35.1 (0.8)
II-III	13	169.1 (20)	10.0 (1.5)	0.50 (0.03)	-34.9 (1.3)
V	13	177.9 (11)	9.6 (0.5)	0.48 (0.02)	-34.3 (0.8)
VI	6	232.8 (55)	7.9 (1.17)	0.52 (0.1)	-37.9 (2.9)

NFS

Layer	*n-number*	*Ir*	*Tm*	*Spike width*	*Spike threshold*
All	62	331 (13)	21.3 (1.0)	0.96 (0.03)	-36.3 (0.5)
II-III	43	341 (16)	22.3 (1.2)	0.96 (0.04)	-36.2 (0.7)
V	13	311 (19)	20.6 (1.1)	0.95 (0.06)	-37.6 (0.7)
VI	6	285 (39)	14.4 (1.7)$+	0.91 (0.02)	-33.4 (0.92)$+

* FS vs NFS
# LII-III vs LV
$ LVI vs LII-III
+ LVI vs LV

Supplemental Table 1. Passive and active properties of fast-spiking and non-fast-spiking interneurons. Ir = input resistance, Tm = membrane time constant.

Distributed network actions by nicotine increase the threshold for spike-timing-dependent plasticity in prefrontal cortex

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Chapter 4

Published in Neuron 2007 Apr 5; 54(1): 73-87

Abstract

Nicotine can enhance attention and working memory in primates and rodents by activating nicotinic acetylcholine receptors (nAChRs). The prefrontal cortex (PFC) is critical for these cognitive functions and is also rich in nAChR expression. Nonetheless, the specific cellular and synaptic mechanisms underlying nicotine's beneficial effects on cognition remain elusive. During cortical function, the strength of excitatory glutamatergic synapses is thought to be regulated through Hebbian-like mechanisms. Here we show that nicotine exposure can increase the threshold for synaptic spike-timing-dependent potentiation (STDP) in layer V pyramidal neurons of the mouse PFC. During coincident presynaptic and postsynaptic activity, nicotine reduces dendritic calcium signals associated with action potential propagation by enhancing GABAergic transmission. This results from a series of presynaptic actions involving different PFC interneurons and distributed mechanisms of modulation by multiple nAChR subtypes. Pharmacological block of nAChRs or GABAA-Rs prevented nicotine's actions and restored STDP, as did increasing dendritic calcium signals with stronger postsynaptic activity. Thus, by activating nAChRs distributed throughout the PFC neuronal network, nicotine has an impact on PFC information processing and storage by increasing the amount of postsynaptic activity necessary to induce STDP, an effect that outlasts nAChR stimulation.

Introduction

Nicotine is the addictive ingredient in tobacco that drives people to dependence, but it has also been shown to improve cognitive function in humans and laboratory animals (Levin et al., 2005; Levin, 1992; Mansvelder et al., 2006; Newhouse et al., 2004b). In smokers and patients suffering from a variety of neuropsychiatric disorders, nicotinic agonists act beneficially on several aspects of cognition, including working memory, attention, learning and memory. In fact, nicotinic treatments are being developed as therapy for cognitive dysfunction in disorders such as Alzheimer's disease, Parkinson's disease, schizophrenia and ADHD (Levin et al., 2005; Newhouse et al., 2004a; Newhouse et al., 2004b; Picciotto and Zoli, 2002). In contrast, in normal nonsmokers, nicotine tends to have deleterious effects on cognitive performance (Newhouse et al., 2004b). Although it is likely that many brain areas contribute to the nicotinic effects on cognition, based on animal studies nicotinic acetylcholine receptors (nAChRs) in the prefrontal cortex (PFC) mediate the effects on attention and working memory performance (Granon et al., 1995; Levin, 1992; Muir et al., 1995). The rodent medial PFC is considered to be functionally homologous to the primate dorsolateral PFC and has been shown to be involved in attention and working memory (Dalley et al., 2004; Groenewegen and Uylings, 2000). Despite this understanding of nicotinic effects on working memory and attention performance, very little is known of the cellular and synaptic mechanisms involved in the enhancement of these functions.

Excitatory glutamatergic synapses in the PFC are plastic and changes in synaptic strength occur in the rodent PFC during working memory-related tasks (Jay et al., 1995; Laroche et al., 2000; Laroche et al., 1990). Changes in strength of cortical synapses are thought to occur depending on the precise timing of pre- and postsynaptic activity, a process known as spike-timing-dependent plasticity (Bi and Poo, 1998; Magee and Johnston, 1997; Markram et al., 1997). The relative timing of pre- and postsynaptic activity results in specific postsynaptic changes in calcium concentration that determine whether synaptic strength will increase or decrease (Koester and Sakmann, 1998; Sjostrom and Nelson, 2002). Nicotinic AChRs are ligand-gated cation channels that — depending on their subcellular location — can alter presynaptic release of neurotransmitters, as well as alter somatic or dendritic membrane potential (MacDermott et al., 1999; McGehee and Role, 1995). These nicotine-induced cellular and synaptic alterations have been shown to affect the induction of long-term changes in synaptic strength in the ventral tegmental area (VTA) and hippocampus (Ge and Dani, 2005; Ji et al., 2001; Mansvelder and McGehee, 2000). These and other studies highlight that in order to understand nicotinic modulation of information processing in a particular brain area, one needs to understand how nicotine affects the different cell types in the neuronal network. More specifically, nicotinic modulation of a neuronal network depends on 1) the types of neurons in the network that express nAChRs; 2) the types of nAChRs expressed; and 3) the subcellular location of these

nAChRs (Alkondon and Albuquerque, 2004; Ji et al., 2001; MacDermott et al., 1999; Mansvelder and McGehee, 2002; McGehee and Role, 1996; Wonnacott et al., 2005). None of these aspects have been addressed in the PFC (Gioanni et al., 1999; Lambe et al., 2003; Mansvelder et al., 2006; Vidal and Changeux, 1989; Vidal and Changeux, 1993).
Therefore, to understand the synaptic and cellular mechanisms underlying nicotinic enhancement of PFC-based cognition, we investigated how nicotinic modulation of the PFC neuronal network affects STDP. We find that nicotine increases the threshold for induction of STDP in pyramidal neurons. This effect is caused by a reduction of dendritic calcium signaling in these neurons as a result of nicotine-induced augmentation of GABAergic inhibition. Our study also demonstrates that both specific classes of PFC interneurons express nAChRs, and that specific inputs to these cell types in the medial PFC neuronal circuitry are modulated by nAChR stimulation. By affecting different parts of the PFC neuronal network through activating different nAChR types, nicotine raises the threshold for the induction of STDP in PFC output neurons.

Materials and methods

Slice preparation
Prefrontal coronal cortical slices (300 μm) were prepared from P14-23 C57 BL/6 mice, in accordance with Dutch licence procedures. Brain slices were prepared in ice-cold artificial cerebrospinal fluid (ACSF) which contained in (mM): NaCl 125; KCl 3; NaH2PO4 1.25; MgSO4 3; CaCl2 1; NaHCO3 26; glucose 10; 300mOsm. Slices were then transferred to holding chambers in which they were stored in ACSF which contained (in mM): NaCl 125; KCl 3; NaH2PO4 1.25; MgSO4 2; CaCl2 1; NaHCO3 26; glucose 10, bubbled with carbogen gas (95% O2/ 5% CO2).

Electrophysiology
Pyramidal cells and interneurons in the medial PFC were first visualized using Hoffman modulation or infrared differential interference contrast microscopy. After the whole cell configuration was established, recorded responses to steps of current injection allowed us to classify each cell as one of several well known cortical cell types. In many experiments two-photon imaging was also used to produce an overview of the cell's morphology. All experiments were performed at 31-34 °C.
Recordings were made using Axopatch or Multiclamp 700A amplifiers (Axon Instruments, CA, USA) sampling at intervals of 50 or 100 μs, digitized by the pClamp software (Axon), and later analyzed off-line (Igor Pro software, Wavemetrics, Lake Oswego, OR, USA). Whole cell current injection and extracellular stimulation (both timing and levels) were controlled with a Master-8 stimulator (A.M.P.I., Jerusalem, Israel) triggered by the data acquisition software. Patch pipettes (3-5 MOhms)

were pulled from standard-wall borosilicate capillaries and were filled with one of three intracellular solutions. For the measurement of EPSCs in pyramidal cells and interneurons we used a solution containing (in mM): K-gluconate 140; KCl 1; HEPES 10; K-phosphocreatine 4; ATP-Mg 4; GTP 0.4, pH 7.2-7.3, pH adjusted to 7.3 with KOH; 290-300 mOsm. The chloride concentration in this intracellular solution was chosen so that the calculated chloride reversal potential was far below the resting membrane potential (-120 mV) and IPSCs would show as outward current in the recording, while EPSCs would show as inward current. This solution was not used in experiments looking specifically at GABAergic activity, where we used an elevated chloride concentration (potassium gluconate 78 mM, KCl 70 mM) to make detection of GABA events more reliable. For the STDP experiments, we used a solution that had a lower osmolarity (potassium gluconate 110 mM, KCl 10 mM; 270-275 mOsm) and an elevated phosphocreatine (10 mM) concentration. Series resistance was not compensated.

For the experiments in Figures 2C and 5D-F, nicotine was applied by pressure ejection from a glass electrode with a tip opening of ~1 µm. Pressure was on for 100 ms. Care was taken that nicotine-containing solution did not leak out of the electrode when no positive pressure was applied by including Alexa488 (100 µM) in the application pipette so that any leaking solution could be easily visualized using two photon microscopy. During application, the extent of application was visualized by monitoring the green fluorescence signal of Alexa488 in some of the experiments.

In the experiments in Figure 3A we blocked GABAA receptors to investigate the involvement of GABAergic transmission in the effects of nicotine on STDP. Both bicuculline (1-10 µM) and Gabazine (0.25-1 µM) strongly increased the excitability of the PFC network. In some slices, excitation was so strong that spontaneous seizure-like network discharges appeared (Suppl Fig 4B). During these discharges, pyramidal neurons received a barrage of synaptic inputs and their membrane potential depolarized by 20-40 mV. In addition, the intracellular calcium concentration in dendrites increased markedly (Suppl Fig 4C, D). To prevent these bursts from compromising our experiments in any way, we only analyzed experiments during which no network discharges appeared.

Spike-timing-dependent plasticity

EPSPs were evoked every 10s using an extracellular stimulation electrode positioned in L2/3 (Fig 1A), ~ 100 µm lateral to the recorded pyramidal cell. The slope of the initial 2.5 ms of the EPSP was analyzed to ensure that the data reflected only the monosynaptic component of each experiment (Froemke et al., 2005). Synaptic gain was measured as the change in average EPSP slope when comparing a five minute period 20-30 minutes post-conditioning to the baseline EPSP slope measured in the last 5 minutes of control recording. During the induction protocol spike-timings were measured from the onset of the evoked EPSP to the peak of the postsynaptic AP. Mean

baseline EPSP slopes were averaged from at least 30 sweeps. During the conditioning period pre-postsynaptic stimulus pairing was repeated 50 times, with a 10s interval between each pairing. An interval between presynaptic stimulation and postsynaptic action potential of 5 ms resulted in reliable potentiation of synaptic strength under control conditions (Fig 1). During experiments cell input resistance was monitored throughout by applying a 10 pA, 500 ms hyperpolarizing pulse at the end of each sweep. Subtle changes in series resistance were usually first detected as a change in the evoked AP waveform, and experiments were not included in the analysis if the cell input resistance varied by more than ±30% during the experiment. To assess the effect of nicotine in these experiments, nicotine was applied during the induction phase (1 minute before through +3 minutes after start of the 8 minute pairing protocol). The Wilcoxon's signed rank test and the Mann-Whitney U-test were used to assess significance. Data are given as mean ± SEM, with $p< 0.05$ indicating significance.

Two-photon imaging

Pipettes were tip-filled with intracellular medium and back-filled with intracellular solution containing Alexa 594 (40 μM) to reveal neuronal morphology and the calcium-indicator Fluo-4 (100 or 200 μM, Molecular Probes). Following breakthrough, cells were monitored for a minimum of 15 minutes to allow diffusion and equilibration of the dye intracellularly before fluorescence measurements were taken. Responses were measured to individual APs or 2 to 3 APs ("burst") during a 20 msec period of current injection. Somatic APs were observed during all line scans analyzed in this data set. To stimulate EPSPs, an extracellular stimulation electrode was placed within 100 μm laterally to the region of apical dendrite being line-scanned in layer II/III. Approximately five minutes after baseline linescan measurements in aCSF were made, the dendritic region of interest (ROI) was moved approx. 5 μm closer to the soma and further linescans were taken in either aCSF or following bath-application of nicotine (10 μM).

A Leica (Mannheim, Germany) RS2 two-photon laser scanning microscope was used with a x63 objective and with a Ti:Sapphire laser (Tsunami, SpectraPhysics, CA, USA) tuned to a wavelength of 840 nM for excitation. Line-scan imaging of spines and dendrites was carried out at a temporal resolution of 2 msec/line. Line-scan imaging and electrophysiological recordings were synchronized and all image acquisition was controlled by custom-written macros based on Leica confocal software. Fluorescence was measured across the apical dendrite at a distance of approximately 110 ± 6 μm from the soma. Before stimulation, fluorescence was measured for approx 85 msec to obtain basal fluorescence measurements (Fo). A region of line scan outside of any indicator-filled ROI was used to measure background fluorescence (Fb). Relative fluorescence changes were calculated as follows: $\Delta G/R=(F(t)-Fo)/(Ro-Rb)$, where Ro is the baseline signal measured with Alexa594 and Rb is the background signal measured in this channel. $\Delta F/F$ and $\Delta G/R$ signals were measured by detecting the

peak change in a trace, averaging a 10 millisecond region around the peak change and expressing it as a percentage change from baseline level. Fluorescence traces for single APs and bursts of APs are averages of 3-5 traces. Offline data analysis was carried out using in house written procedures in Igor Pro software. Differences between groups were tested using ANOVA and t-tests (paired or two-tailed independent samples) in SPSS statistical software with $p< 0.05$ indicating significance.

Single-cell RT-PCR

For single-cell analyses, we used the same solution as was used for the measurement of spontaneous activity. After recordings were made, the cell was aspirated and the content (~7.5 µl) was expelled in a tube containing 2 µl RT-buffers (final concentrations: 10 mM DTT, 0.5 µM dNTP, 5 µM random hexamers). An enzyme mixture was added (0.5 µl, containing: 50 U MMLV (Promega); 5 U RNAguard (Amersham)), and after gently mixing, the reaction was performed O/N at 37 °C. After precipitation (final concentration: 2 M NH4Ac, 75% EtOH, 0.1 µg linear acrylamide) on ice for 30 min, samples were spun (14,000 g, 30 min), washed twice with 75% EtOH, and collected in 10 µl water. Reactions were stored at 4° C.

For PCR analysis, a pre-amplification (nAChR subunits, 25 cycles; others, 15 cycles) was followed by amplification (45 cycles) using real-time PCR (10 µl; ABI PRISM 7900, Applied Biosystems). Nested primer sets (Suppl. table 1) were used for amplification. Whereas the pre-amplification of GAD, calbindin, cholecystokinin and somatostatin was carried out in separate reactions (triplicate) using 0.3 µl cDNA, preamplification of the nAChR subunits was performed with a mixture of primers for the α4, α7, and β2 subunits (triplicate) using 1.5 µl cDNA. The amplification was carried out on 2% volume of the pre-amplification reaction. For each transcript analyzed, a negative control (H2O) reaction was performed. For both reactions, the SYBR-reagents (2x SYBR-mix; Applied Biosystems) were used, with the following PCR parameters: 10 min at 95 °C, followed by 45 cycles using 15 sec at 95 °C, 1 min 60 °C. For each primer, a positive control (1:10,000 dilution of mouse PFC cDNA) and a negative control (H2O) were used. At the end of each PCR, a dissociation stage was performed in order to check for specificity of the formed product (from 60 ° to 95 °C in 15 min; Suppl figure 5). A single PCR round (45 cycles) using β-actin was performed to detect the formation of cDNA, resulting in 52 cells positive out of 54.

Results

Nicotine blocks spike-timing-dependent potentiation of glutamatergic transmission

Since nicotine alters cognitive performance of rodents in behavioral tasks that involve PFC function (Granon et al., 1995) and changes in excitatory synapse strength occur during such tasks (Jay et al., 1995; Laroche et al., 2000; Laroche et al., 1990), we asked whether nicotine alters synaptic plasticity in medial PFC. To test this, we made whole-

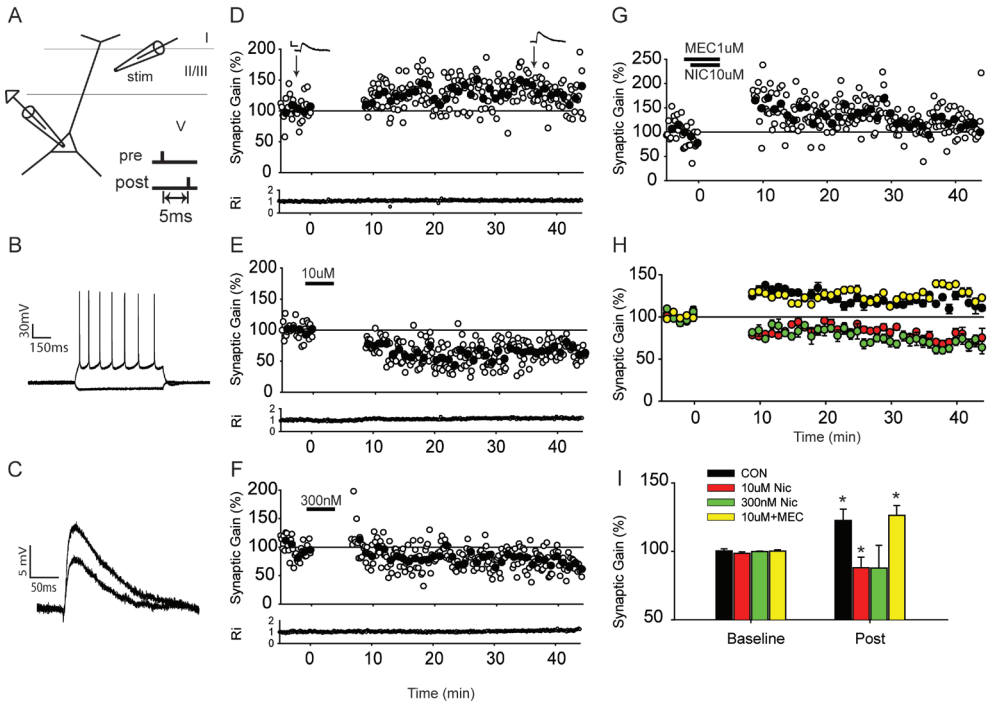


Figure 1. Spike-timing-dependent potentiation in layer V pyramidal cells of the mouse prefrontal cortex.

(A) Graphic scheme of experimental set-up depicting placement of extracellular stimulating electrode and timing of STDP induction protocol (inset).

(B) Representative current clamp traces from a layer V pyramidal cells (injection of -100 and +50pA).

(C) Example EPSPs recorded before (smaller) and after (larger) STDP induction.

(D-G) Example experiments showing spike-timing-dependent potentiation in a layer V pyramidal cell for (D) control, (E) 10 μM nicotine, (F) 300nM nicotine and (G) nicotine (10 μM) and mecamylamine (MEC, 1 μM). Duration of applications is indicated by the bars above the graphs. Synaptic gain at 35-45 minutes after induction is significantly above baseline ($p<0.05$), in line with the average shown in H. H Average temporal plot comparing change in EPSP slope in control (black circles; n=6), nicotine (10 μM; red circles; n=7, 300nM: green circles; n=5) and nicotine with MEC (yellow circles; n=8).

(I) Bar graph summarizing STDP in control, nicotine, and MEC. Statistical significance indicated * for $p<0.05$.

cell recordings from layer V pyramidal neurons and stimulated glutamatergic inputs by extracellular stimulation (Fig 1A). Pyramidal neurons were identified based on morphological appearance in the DIC image and action potential profile in response to step depolarizations (Fig 1B). To induce spike-timing-dependent potentiation (STDP), extracellular stimulation of presynaptic glutamatergic input was paired with a single postsynaptic action potential evoked by somatic current injection (Fig 1A, inset). Repeated pairing of the EPSP with a single postsynaptic action potential (5 ms after start of EPSP, 50x at 0.1 Hz) resulted in a lasting increase of both EPSP amplitude

and slope (increase slope: 133.3±19.7%; Fig 1D, H).
When nicotine (10 μM) was applied briefly during the start of the pairing period, glutamatergic synaptic strength failed to increase (Fig 1E, H). Instead, nicotine induced a small and significant reduction in synaptic weight to 87.0±10.3% of control after induction (Fig 1H, I). During cigarette smoking, blood levels of nicotine rapidly increase to 300-500 nM (Henningfield et al., 1993). Application of 300 nM nicotine during pairing of pre- and postsynaptic activity also prevented the increase of synaptic weight (n=5, Fig 1F, H). Similar to 10 μM nicotine, application of 300 nM nicotine tended towards a reduction of synaptic strength (Fig 1H, I), but this did not reach significance. The broad-spectrum nicotinic receptor antagonist mecamylamine (MEC, 1 μM) prevented the effect of nicotine (Fig 1G, H). In the presence of both MEC and nicotine, the pairing of EPSP and postsynaptic action potential induced a synaptic gain that was indistinguishable from control conditions (n=8, Fig 1H, I). Thus, activation of nicotinic receptors in medial PFC prevents STDP of inputs (evoked in layer II/III) to layer V pyramidal cells and induces LTD instead.

Direct nicotinic actions on evoked glutamatergic transmission
The most straightforward mechanism by which nicotine alters synaptic plasticity is through activation of either postsynaptic nAChRs, or presynaptic nAChRs on the glutamatergic terminals. In other brain areas, activation of nAChRs located on presynaptic glutamatergic terminals increases release of glutamate directly (Gray et al., 1996; McGehee et al., 1995). In VTA, activation of these presynaptic nAChRs can induce LTP (Mansvelder and McGehee, 2000). In PFC, nicotine also augments spontaneous excitatory glutamatergic synaptic transmission to layer V pyramidal neurons by activating presynaptic nAChRs (Gioanni et al., 1999; Lambe et al., 2003; Vidal and Changeux, 1989; Vidal and Changeux, 1993). This effect depends on action potential firing, indicating that nAChRs are located away from the presynaptic terminals. In our hands, nicotine also induced an increase in frequency and amplitude of spontaneous excitatory postsynaptic currents (EPSCs, Fig 2A, B). The EPSCs disappeared in the presence of the AMPA-R blocker DNQX (10 uM, n=4, Suppl Fig 1A, B). Because nicotine's effect on spontaneous release of glutamate was opposite to its effect on STDP, we next tested if nicotine affected evoked glutamatergic transmission by stimulating either in layer II/III or in layer V, while recording from layer V pyramidal neurons (Fig 2D). Evoked EPSCs resulting from either stimulating layer II/III or layer V were not increased in amplitude by nicotine (Fig 2E, F). Instead, evoked EPSC amplitude showed a small transient reduction that hardly outlasted the nicotine application (Fig 2F). Furthermore, puffing nicotine directly onto pyramidal neurons did not elicit an inward current (n=15, Fig 2C), suggesting that like in other neocortical areas, PFC layer V pyramidal neurons do not express functional nAChRs. These data demonstrate that activation of presynaptic glutamatergic inputs or postsynaptic nAChRs on pyramidal neurons cannot explain the effect of nicotine

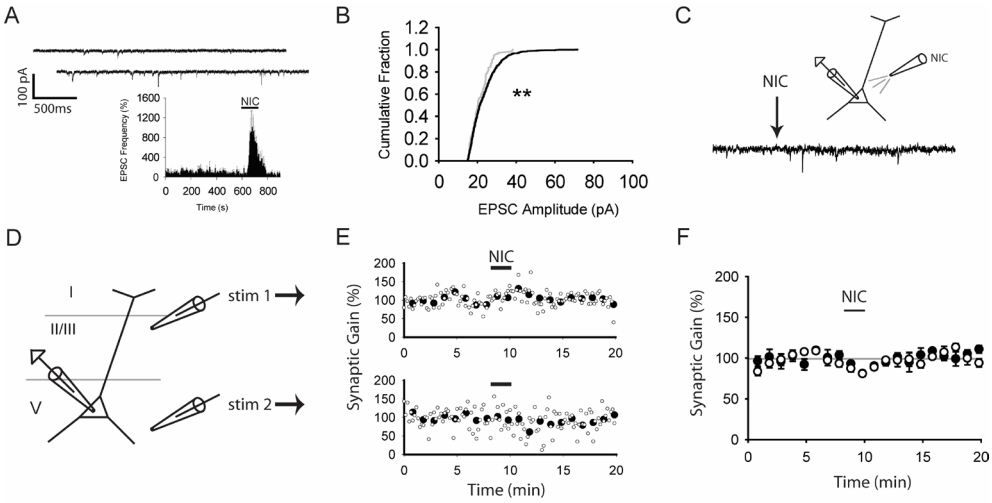


Figure 2. Glutamatergic inputs to layer V pyramidal cells.
(A) Example trace with spontaneous EPSCs recorded in layer V pyramidal cell. Top trace: control; Lower trace: in the presence of nicotine (10 μM). Scale bar 100pA, 500ms. Inset below: frequency histogram for spontaneous EPSCs (n=6).
(B) Cumulative EPSC amplitude distribution before (grey) and after (black) nicotine application. Data is taken from experiment shown in A.
(C) Voltage clamp trace from a layer V pyramidal cells where nicotine was locally applied at the arrow and no current was observed (n=14). Inset above shows experimental set-up.
(D) Experimental set-up for E. E Normalized amplitude of evoked EPSPs (grey circles) and mean amplitude per minute (black circles) recorded from the two stimulation locations depicted in D.
(F) Average plot of evoked EPSP experiments from layer II/III (white circles) and layer V (black circles) (n=6).

on STDP in PFC.

Blocking GABAA receptors but not GABAB receptors, strongly reduces nicotine's impact on STDP

An alternative mechanism by which nicotine could affect STDP in PFC is through activation of nAChRs, either on the terminals or somata of inhibitory GABAergic neurons. Indeed, in many cortical and sub-cortical brain areas, nicotine affects not only glutamatergic but also GABAergic synaptic transmission (Alkondon and Albuquerque, 2004; Dani and Harris, 2005; Mansvelder et al., 2006; Mansvelder et al., 2002; Metherate, 2004). In hippocampus, timed activation of GABAergic interneurons by nicotine diminishes or prevents long-term potentiation in pyramidal neurons (Ji et al., 2001). To investigate whether GABAergic transmission mediates nicotine's effect on STDP, we tested how the GABAA receptor blocker Gabazine and the GABAB receptor blocker CGP-54626 affected nicotine's impact on STDP. In the presence of Gabazine (0.25 – 1 μM), the reduction of STDP by nicotine was much less than with nicotine alone (n=8, $p<0.05$; Fig 3A, C). In contrast, CGP-54626 (5 μM) did not affect the block of potentiation by nicotine (n=5, Fig 3B, C). From these results indicate that increased

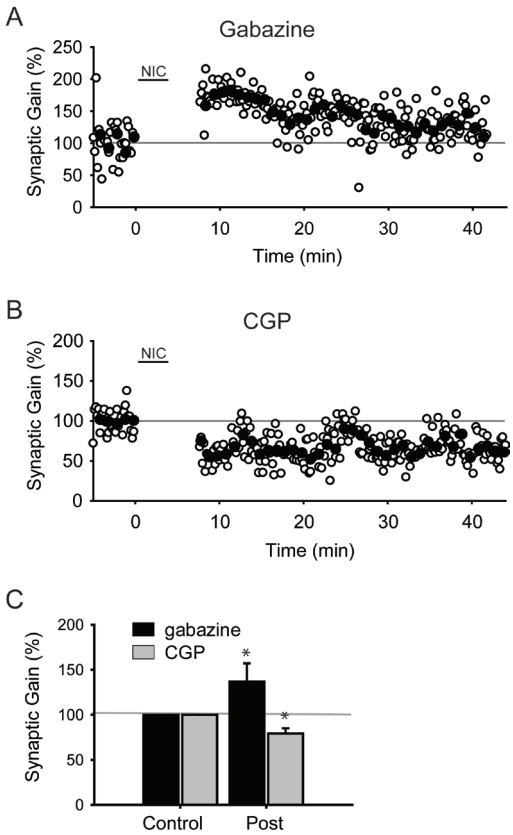


Figure 3: Effect of GABAergic inhibition on spike timing-dependent LTP.
(A) Example trace of spike timing-dependent potentiation in a layer V pyramidal neuron in the presence of 10μM nicotine and the GABAA antagonist, gabazine (1 μM)
(B) Example trace of blocked potentiation in the presence of 10 μM nicotine and the GABAB receptor blocker CGP-54626 (5μM).
(C) Summary bar graph. * indicates $p<0.05$.

GABAergic signaling through GABAA receptors mediates nicotine's block of spike-timing-dependent potentiation in PFC.

Nicotine enhances inhibitory GABAergic transmission to pyramidal neurons

To assess to what extent nicotine affects inhibitory GABAergic transmission received by layer V pyramidal neurons, inhibitory postsynaptic currents were evoked by stimulating layer II/III while recording from layer V pyramidal neurons. The amplitude of evoked IPSCs was transiently enhanced by nicotine (Fig 4A, B). On average nicotine increased the GABAergic synaptic strength by 141±11%, which subsided when nicotine was washed out (Fig 4B). Nicotine also affected spontaneous inhibitory synaptic transmission. Both frequency and amplitude of spontaneous IPSCs strongly increased when nicotine (10 μM) was bath applied in all cells tested (n=7, Fig 4C-E). IPSC frequency was increased to 246±72%. Low nicotine concentrations (300 nM) also augmented spontaneous IPSC frequency by 131.5 ± 3% (n=5, Fig 4F). IPSC amplitude distribution was increased in all cells tested. Cumulative amplitude distributions showed that nicotine had a strong effect on larger amplitude synaptic currents (Fig 4E). IPSCs disappeared when bicuculline (10 μM) was applied (n=3, Suppl Fig1C, D) and nicotine's effect was blocked by TTX (n=3, Suppl Fig 2B). These experiments show that in addition to augmenting excitatory synaptic transmission to PFC pyramidal neurons, inhibitory GABAergic transmission is also enhanced by nicotine.

As an initial pharmacological characterization of the nAChRs subtypes involved in augmenting spontaneous GABAergic transmission in PFC pyramidal neurons, we tested the effect of nicotine in the presence of MEC and methyllycaconitine (MLA),

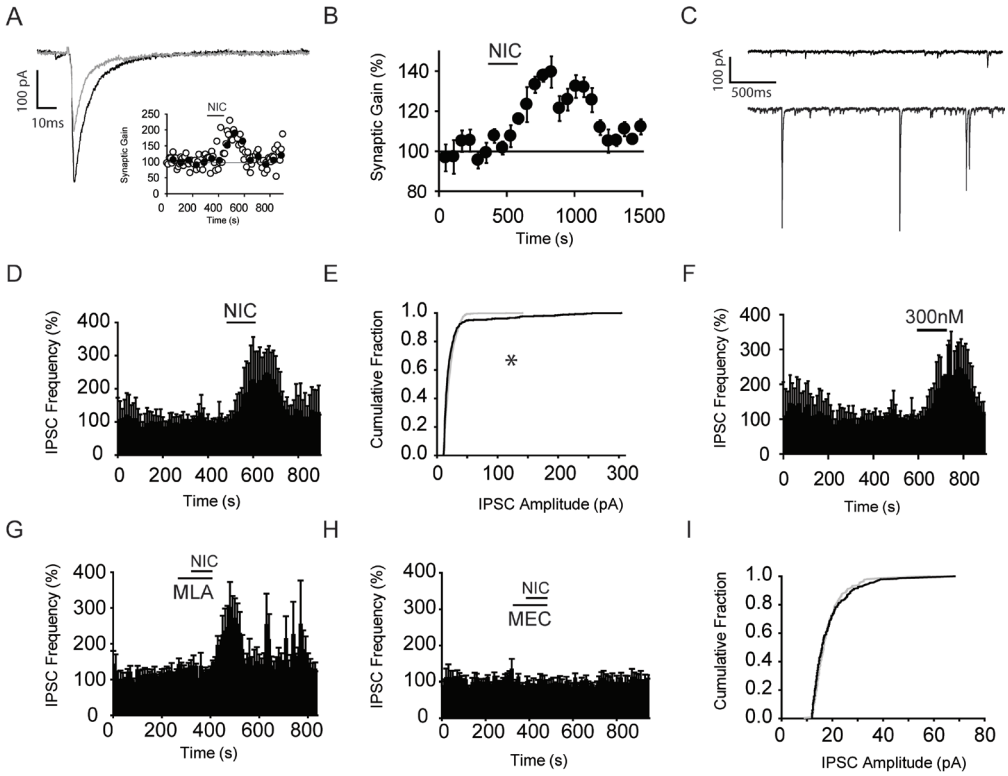


Figure 4. GABAergic transmission to layer V pyramidal cells.
(A) Example evoked IPSCs before (black trace) and after (red trace) nicotine application. Inset, Temporal plot of normalized amplitude (white circles) and mean per minute (black circles) from a single experiment.
(B) Summary of evoked IPSC experiments (n=6).
(C) Example spontaneous IPSCs recorded from a layer V pyramidal cell in the absence (top trace) and presence of 10 μM nicotine.
(D) Average IPSC frequency histogram (n=7).
(E) Cumulative IPSC amplitude distribution from experiment shown in D ($p<0.001$).
(F) Average IPSC frequency histogram with 300nM nicotine application (n=5)
(G) Average IPSC frequency histogram (n=4). Duration of MLA and nicotine application is indicated by bars above graph.
(H) Average IPSC frequency histogram with the application of MEC and nicotine (n=7).
(I) Cumulative IPSC amplitude distribution from a single MEC/NIC experiment ($p=0.3$).

which is more selective for α7-containing nAChRs. In the presence of MLA (10 nM), nicotine still increased the frequency of spontaneous IPSCs but not in all cells tested. In 3 out of 4 cells, nicotine increased the IPSC frequency to 294±18% (Fig 4G). The IPSC amplitude distribution was also shifted to larger amplitudes in 3 out of 4 cells (Suppl Fig 2A). This suggests that MLA sensitive nAChRs do contribute to the effect of nicotine on spontaneous IPSCs. In the presence of MEC (1 μM), nicotine application did not affect the frequency of spontaneous IPSCs in 5 out of 7 cells. Also the effect of

nicotine on amplitude distribution was blocked in 5 out of 7 cells (Fig 4H, I). Nicotine most likely activates multiple types of nAChRs to increase both frequency and amplitude of spontaneous IPSCs and augment inhibition of PFC pyramidal neurons.

Nicotine excites different types of interneurons through multiple mechanisms

To delineate which types of interneurons express functional nAChRs, we targeted different classes of interneurons for whole-cell recording. In rat PFC several types of pyramidal neurons and interneurons have been described based on electrophysiological profile, morphology and expression of calcium-binding proteins (Gabbott et al., 1997; Gulledge et al., 2007; Kawaguchi, 1993; Kawaguchi and Kondo, 2002; Kawaguchi and Kubota, 1997; Yang et al., 1996). A characterization of interneurons in mouse medial PFC has not been described in the literature thus far, and we identified three different classes of interneurons based on action potential firing profile in response to current steps (Fig 5A-C). Their morphological appearance was clearly distinct from the typical layer V pyramidal neuron morphology (Fig. 5A-C insets).

The first type of interneuron had the typical characteristics of fast spiking (FS) interneurons in other cortical areas (Kawaguchi and Kondo, 2002). They were multi-polar with round cell bodies. In response to step current injections they showed non-adapting, tonic firing behavior with a firing frequency that was proportional to the amount of depolarizing current injection (Fig 5A). Local puffs (100 ms) of nicotine to the cell body region of this type of interneuron did not elicit inward currents in any of the cells tested (n=24; Fig 5D, G, H).

The action potential profile of a different type of interneuron showed slight adaptation in response to step current injections (Fig 5B). These cells had a multi-polar appearance similar to FS cells. These cells were named regular spiking non-pyramidal neurons (RSNP). In response to local application of nicotine, a fast inward current of 12±6 pA was activated in about half of the RSNP neurons (n= 60; Fig 5E, G, H).

The third type of interneuron showed strong adaptation of firing frequency in response to step depolarizations, and had a lower threshold for firing (Fig 5C). In many cases, these cells fired rebound spikes after a step hyperpolarization. Therefore, these cells were named low-threshold spiking (LTS) cells. As FS cells, LTS cells had a bi-polar appearance or showed in addition to smaller multi-polar dendrites one dendrite of larger diameter that pointed towards layer VI. These cells often also showed a limited number of dendritic spines. Upon application of nicotine to their somatic region a large inward current was activated in all cells tested of 28±10 pA, with a faster rise-time than the nicotine-induced current in RSNP cells (n=10; Fig 5F, G, H). The amplitude of nicotine-induced currents were largest in these cells, and nearly double the amplitude of nicotine-induced currents in RSNP cells (Fig 5H).

During and after pressure application of nicotine, noise levels in recordings from RSNP and LTS cells increased. In RSNP cells (Fig 5E), the noise at the peak of the nicotine-induced current was increased from 29.9±0.026 pA to 52.1±16.0 pA and

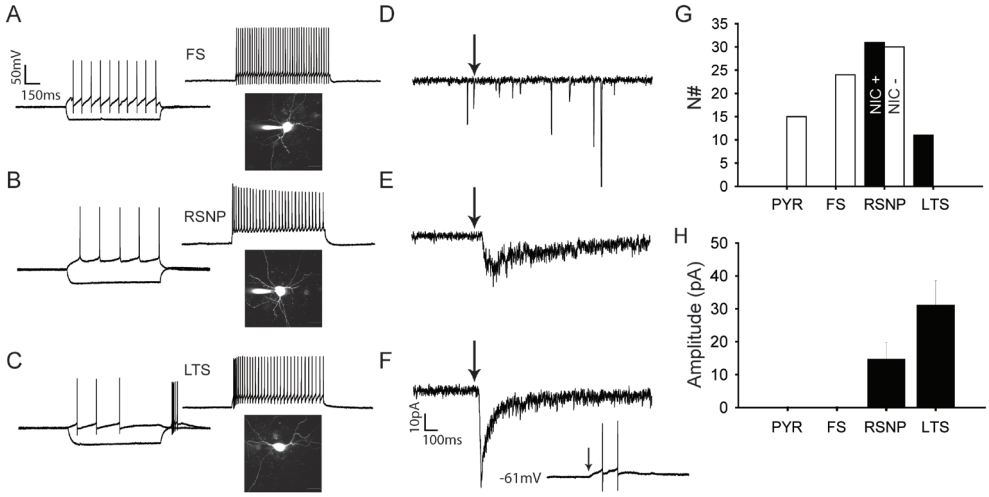


Figure 5. Local application of nicotine onto PFC interneurons.
(A-C) Example current-clamp recordings and morphology of Alexa-filled cells illustrating the three basic interneurons observed in mouse PFC: fast spiking cells (FS), regular spiking non-pyramidals (RSNP), and low-threshold spiking cells (LTS).
(D-F) Example voltage-clamp traces in each of the three cell types depicted in (A) where nicotine was locally applied to the soma (100 ms pressure ejection at arrow).
(F) Inset: current-clamp recording showing that single somatic application of nicotine onto LTS cells can induce spiking.
(G) Histogram comparing the number of cells in each cell class that were positive (black) and negative (white) for nicotinic currents.
(H) Histogram showing average current observed in positive cells from G.

diminished in 4 sec to 42.4±20.9 pA. In contrast, in LTS cells (Fig 5F), the noise at the peak of the nicotine-induced current was not larger than at baseline (29.8±0.018 pA vs 29.7±0.19 pA), but steadily increased during 4 sec to 49.4±1.54 pA at the end of the trace. Most likely, open channel noise from nAChRs contributes to the noise in both neurons, but nicotine might also activate synaptic currents in these neurons.

Different types of interneurons differentially express mRNA for nAChRs
To test which nAChR subunits were expressed by the different types of interneurons we determined the presence of mRNAs for the most abundant nAChRs in the brain, α4, β2, and α7 using single-cell PCR (Cauli et al., 2000; Liss, 2002). After establishing the whole-cell configuration in the PFC slice and applying step current injections to obtain the action potential profile of the interneuron, the cell contents were aspirated into the recording pipette and real-time PCR was performed on the tip contents (Table 1). GAD2 mRNA, encoding the glutamic acid decarboxylase enzyme GAD65, was abundantly detected in all three interneuron types, suggesting that these cells synthesize the neurotransmitter GABA. In addition, expression of genes encoding

different calcium-binding proteins and peptides was detected in these cells. RSNP cells abundantly expressed Calbindin (CB) and cholecystokinin (CCK), but to a lesser extend somatostatin (SOM). In contrast, a much smaller proportion of FS cells expressed CCK, whereas the majority of FS cells expressed CB and SOM. LTS cells expressed CB, CCK and SOM to a similar degree (Table 1). Nicotinic AChR mRNA expression patterns were in line with the responses of interneurons to nicotine puffs to the soma. FS cells did not show inward currents upon nicotine application (Fig 5) and hardly any FS cells showed expression for nAChR mRNA. In contrast, LTS and RSNP cells both showed functional responses to nicotine application to the soma (Fig 5), and in both cell types mRNA encoding nAChR subunits were found (Table 1). The largest number of LTS and RSNP cells expressed α4 subunits, but β2 and α7 mRNA was also found in these cell types. These data are also in line with the finding that the augmentation of spontaneous IPSCs by nicotine has a mixed pharmacological profile (Fig 4G and H, Suppl Fig 2).

Excitatory inputs to different types of interneurons are differentially affected by nicotine

We have shown that the mouse medial PFC harbors at least three classes of interneurons that show similar functional and morphological properties as interneurons found in other cortical areas, but differ in functional nAChR expression. FS cells do not express nAChRs somatically, whereas RSNP and LTS cells express functional nAChRs and contain mRNA for α4, β2 and α7 subunits. These cell types are directly depolarized by nAChR activation expressed on their cell bodies or proximal dendrites, and LTS cells could even be made to spike when nicotine was applied in current clamp (Fig 5F inset). These somatic nAChRs could account for the increase in IPSC frequency and amplitude by nicotine observed in recordings from pyramidal neurons (Fig 4). However, as excitatory inputs to pyramidal neurons are modulated by nicotine, excitatory inputs to interneurons could also be modulated by nAChR activation, which would also contribute to increased GABAergic activity by nicotine. Therefore, we monitored spontaneous EPSCs in whole-cell recordings from the three interneuron types.

Nicotine differentially modulated spontaneous excitatory transmission in medial PFC interneurons. Spontaneous EPSCs in FS cells showed a substantial increase in both EPSC frequency and amplitude (Fig 6A-C). EPSC frequency increased by 360±144%

Table 1. Percentage of Cells Positive for Neuropeptides and nAChR Subunits

	GAD2	CB	CCK	SOM	α4	β2	α7
FS (12)	++	++	–	+	––	–	––
LTS (9)	++	+	+	+	++	+/–	+/–
RSNP (27)	++	++	++	––	++	+	+/–

Cells (% positive) for neuropeptides (GAD2, CB, CCK, and SOM) and nAChR subunits (α4, β2, and α7): 0%–10%, ––; 10%–20%, –; 20%–30%, +/–; 30%–40%, +; 40%–50+%, ++. The number of cells assessed is indicated parenthetically.

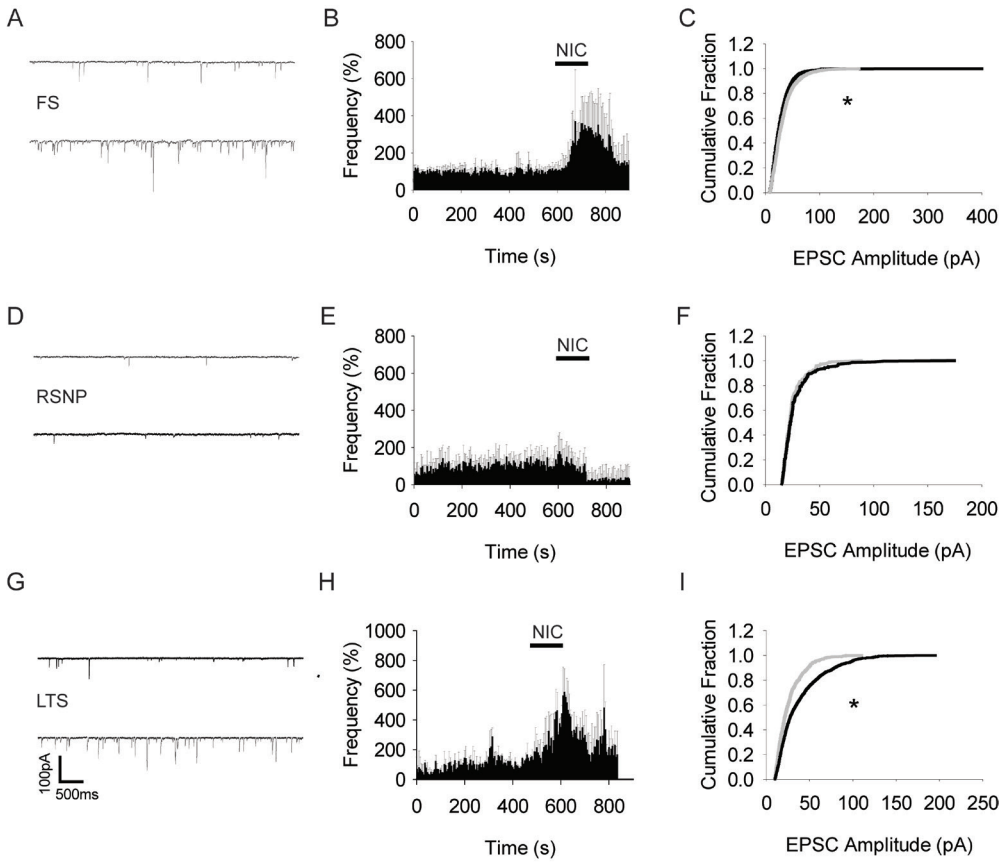


Figure 6. Nicotinic modulation of spontaneous EPSCs received by PFC interneurons.
(A,D,G) Example EPSC traces recorded from (A) fast spiking (FS), (D) regular spiking non-pyramidal (RSNP), and (G) low threshold spiking (LTS) cells respectively.
(B,E,H) Average EPSC frequency histograms for (B) FS, n=7; (E) RSNP, n=11; and (H) LTS cells, n=3.
(C,F,I) Cumulative amplitude distributions for each of the three cell types are shown. Each graph represents data from the experiments depicted in A,D and G. Significance indicated * for $p<0.05$.

and the cumulative distribution of EPSC amplitudes showed that significantly more EPSCs with amplitudes above 25 pA were recruited by nicotine (Fig 6B,C). This effect was blocked by MEC (n=10, Suppl Fig 3A) and TTX (n=6, Suppl Fig 3B). In contrast, spontaneous EPSCs recorded in RSNP cells were negatively modulated by nicotine. EPSC frequency but not amplitude was significantly lower in the presence of bath applied nicotine (Fig 6D-F). Spontaneous EPSCs recorded in LTS cells were increased by nicotine. Both EPSC frequency and amplitude were increased significantly by nicotine application (Fig 6G-I).
Thus, although FS cells do not seem to express functional nAChRs, their excitatory glutamatergic inputs are increased by nicotine, and thus they will receive an increased

excitatory drive in the presence of nicotine. Nicotine will directly depolarize RSNP cells by activating nAChRs on their cell body, but excitatory inputs to these cells are not decreased in amplitude. LTS cells experience an increased excitatory drive from both activated nAChRs on their cell body and an increased glutamatergic input. Our data suggest that all interneuron classes we encountered potentially contribute to the increased inhibition observed in pyramidal neurons in the presence of nicotine.

Dendritic calcium signaling is reduced by nicotine during induction of STDP

Inhibitory GABAergic synaptic transmission controls dendritic action potential propagation in pyramidal neurons. In the hippocampus, IPSPs modulate action potential propagation and calcium signaling, and during development, increased GABAergic inhibition changes the rules for STDP in these neurons (Meredith et al., 2003; Tsubokawa and Ross, 1996). Postsynaptic calcium transients provide an associative link between synapse activation, postsynaptic cell firing and synaptic plasticity (Koester and Sakmann, 1998; Malenka et al., 1988). Since IPSCs in PFC pyramidal cells are increased in amplitude by nicotine (Fig 4), this could reduce dendritic action potential propagation and subsequent calcium signaling in dendrites of PFC pyramidal neurons. We first tested whether changes in intracellular calcium concentration are necessary for STDP in mouse PFC layer V pyramidal neurons. When exogenous calcium chelators such as BAPTA are present in the intracellular solution, incoming calcium ions are rapidly buffered and free calcium concentration changes are strongly reduced (Helmchen, 2002; Tsien, 1980). In the presence of BAPTA (10 mM), pairing pre- and postsynaptic activity did not result in an increase of synaptic strength (n=5, Fig 7A, B). Thus, changes in calcium concentration during STDP induction are necessary for changes in synaptic strength to occur.

To investigate whether nicotine reduced calcium transients related to dendritic action potential propagation, we monitored postsynaptic calcium signaling in apical dendrites of layer V pyramidal neurons during timed pre- and postsynaptic activity. Pyramidal neurons were filled with Alexa594 and the calcium indicator Fluo4 through patch pipettes and were visualized with two-photon imaging to select a region on the apical dendrite for line-scanning (Fig 7C). As in rat PFC (Gulledge and Stuart, 2003), action potentials invade apical dendrites and induce calcium changes throughout the dendritic tree of mouse pyramidal neurons (Fig 7D-F). Somatic action potentials were preceded by extracellular stimulation of synaptic input by 5 ms, as was used for the induction of STDP. Line scans of the apical dendrite were taken 50-100 μm from soma, parallel to the location of extracellular stimulation. After three initial line-scans to obtain baseline measurements, either ACSF or nicotine-containing ACSF was allowed to wash-in for 5 minutes, after which three line-scans were taken (Fig 7D-F right panels). In the presence of nicotine, fluorescence changes of Fluo4 were reduced by approximately 40% when compared to control conditions in the absence of nicotine (Fig 7E-G). Thus, in the presence of nicotine, postsynaptic calcium signals associated with

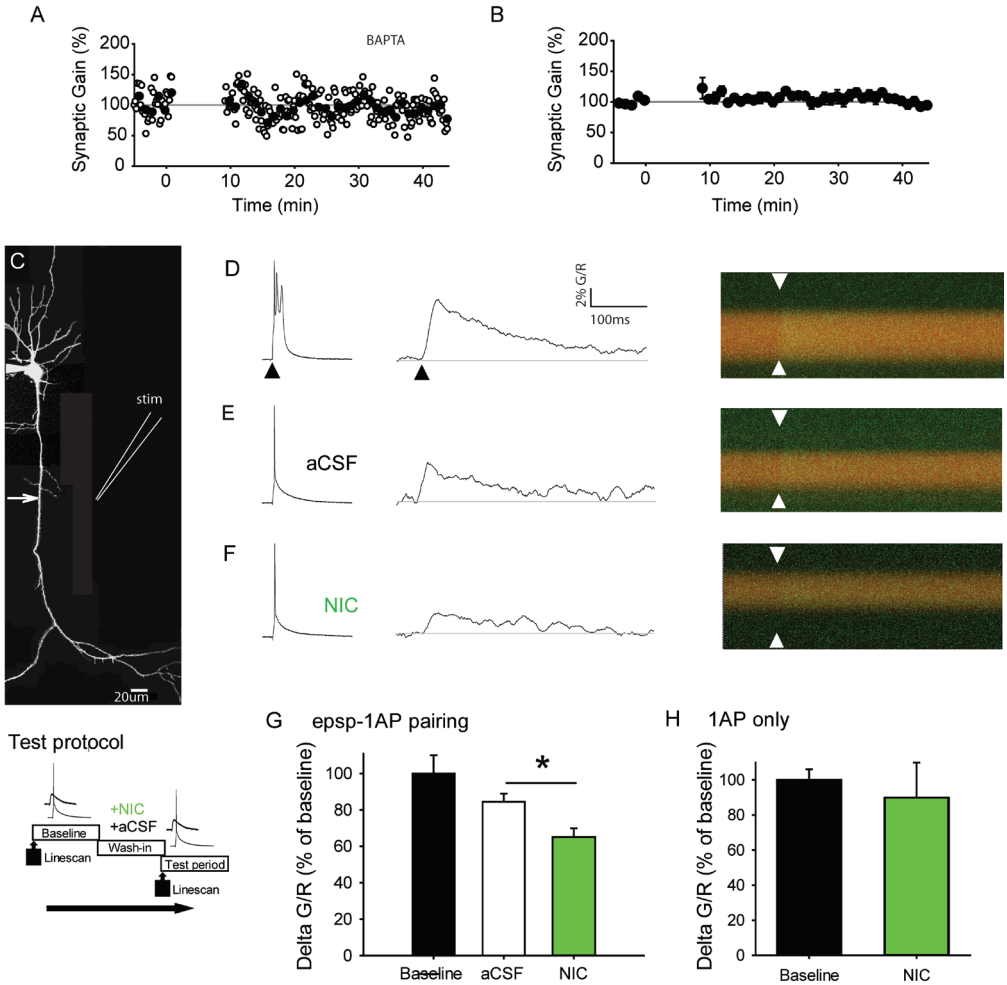


Figure 7. Reduction of AP-induced dendritic calcium signaling in nicotine.
(A) Example trace showing spike-timing-dependent potentiation is abolished in the presence of the calcium chelator BAPTA (10 mM).
(B) Summary of effects of BAPTA on STDP (n=5).
(C) Layer V pyramidal cell filled with Alexa 594 indicating typical position of stimulation electrode and line-scanned ROI (white arrow) Scale bar 20µm . C Lower panel, Experimental stimulation and line-scan protocol.
(D) Example of calcium transient trace in response to AP burst shows clear stimulation-induced change in fluorescence across apical dendrite (middle panel).
(E,F) Example traces of single EPSP-AP induced calcium transients in ACSF (E) and nicotine (F; 10µM) respectively.
(G) EPSP-AP induced calcium transients were significantly lower in the presence of nicotine (64± 6%, n=6) than ACSF (85±4%, n=12, * $p<0.05$).
(H) Nicotine did not induce a significant decrease in AP-induced calcium transients from baseline in the absence of extracellular stimulation (89±20%, n=3, $p>0.3$).

coincident pre- and postsynaptic activity were markedly reduced in apical dendrites of PFC pyramidal neurons. In the absence of extracellular stimulation, nicotine did not alter fluorescence changes associated with postsynaptic action potential firing (Fig 7H). It suggests that PFC pyramidal neurons do not express nAChRs that alter dendritic action potential propagation directly. In fact, during application of nicotine in these experiments, the membrane potential of layer V pyramidal neurons did not depolarize, but instead slightly hyperpolarized by –1.7±0.6 mV. In addition, nicotine did not induce changes in baseline fluorescence. In ACSF conditions, baseline fluorescence increased by 3±2%. In the presence of nicotine, baseline fluorescence increased by 4±1.5%, which was not significantly different from ACSF conditions. These data suggest that dendrites of PFC pyramidal neurons do not contain functional nicotinic receptors that directly affect dendritic calcium signaling.

Inhibition of STDP by nicotine can be overcome by increased postsynaptic activity

Our data suggest that nicotine prevents STDP by decreasing dendritic calcium signaling in pyramidal neurons, not by reducing the calcium signal directly, but indirectly by increasing GABAergic inhibition. It follows then that increased postsynaptic action potential firing might overcome the nicotine-induced augmentation of inhibition and block of synaptic potentiation. To test this, we paired single presynaptic events with short bursts of postsynaptic activity with the same delay of 5 ms (Fig 8A). In the absence of nicotine, short bursts of two or three somatic action potentials during a 20 ms depolarization induced an increase in EPSP slope of 151±15% (Fig 8B, D). When nicotine was applied during pairing of presynaptic events with postsynaptic bursts, the EPSP slope was still increased (n=7, Fig 8C, D). Thus, the block of STDP by nicotine can be overcome by increased postsynaptic activity, which most likely induced larger calcium changes than a single postsynaptic action potential. To confirm that short bursts of action potentials induced increased postsynaptic calcium signaling in the presence of nicotine, we studied dendritic calcium signaling in response to postsynaptic bursts of action potentials. Short bursts of two or three somatic action potentials during a 20 ms depolarization induced larger changes in calcium indicator fluorescence in dendrites than single action potentials (Fig 8E-I). Bath application of nicotine reduced calcium transients induced by single action potentials, but a short burst of action potentials restored calcium signaling (Fig 8I). These data suggest that spike-timing-dependent plasticity in PFC is blocked by nicotine because calcium signaling during dendritic propagation of single action potentials is altered. This block can be overcome by bursts of action potentials that induce larger calcium signals in dendrites, partially restoring spike-timing-dependent plasticity.

Discussion

Nicotinic receptor stimulation alters PFC-based cognitive performance in primates and rodents (Levin et al., 2005; Levin, 1992; Mansvelder et al., 2006; Newhouse et

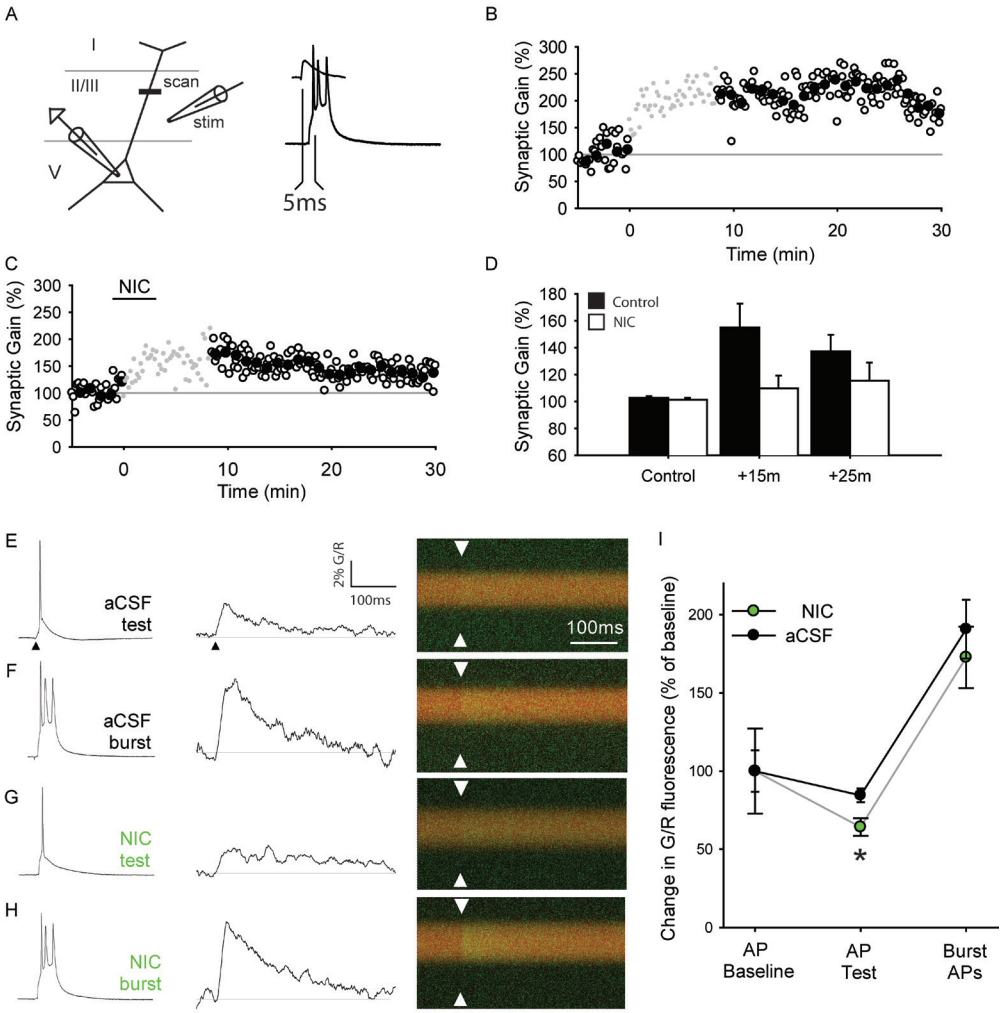


Figure 8. AP bursts rescue nicotine-impaired synaptic plasticity and increase calcium signaling.
(A) Diagram of experimental set-up. Time delay between EPSP and postsynaptic action potential burst of 5ms during STDP induction is illustrated.
(B,C) Normalized EPSP slope (white circles) and mean slope per minute (black circles) recorded from single experiments in control (B) and nicotine (C).
(D) Summary bar graph of potentiation following EPSP-burst pairing stimulation at 15 min and 25 min after induction.
(E-H) EPSP-AP burst pairings induced significantly larger dendritic calcium transients in layer V pyramidal neurons in both the absence (F) and presence of nicotine (H) than compared with EPSP-single AP pairings in similar conditions (E,G, respectively; ACSF: n=12 pairwise comparisons, $p<0.05$; NIC: n=6, $p<0.05$). Arrow heads indicate time point of stimulation. I Nicotine induces a significantly greater decrease in fluorescence following EPSP-single AP pairing compared with ACSF conditions (NIC: 64± 6%, n=6; ACSF: 85±4%, n=12, independent samples t-test, * $p<0.05$, data from same experiments as in figure 7G), but shows similar increase in EPSP-AP burst-induced calcium transients to those in ACSF ($p=0.5$).

al., 2004b). In this study, we find that during nAChR activation the timed pairing of presynaptic activity with single postsynaptic action potentials in pyramidal neurons is no longer sufficient to induce long-term potentiation of excitatory synapses. Increased postsynaptic activity can overcome this blockade of spike-timing-dependent potentiation. During nAChR activation dendritic calcium signaling is reduced, most likely due to increased inhibitory synaptic transmission to pyramidal neurons. Activity of different types of PFC interneurons is increased by nicotine, and we find that different mechanisms are involved (Fig 9). Some interneuron types express nAChRs somatically, such as RSNP and LTS cells. Single-cell PCR data suggest that both cell types express the abundant subunit types α4, β2 and α7. FS interneurons do not express nAChRs somatically, but excitatory inputs to these neurons are augmented by nAChR stimulation. Very little mRNA for α4, β2 and α7 subunits was found in these cells. Excitatory inputs to LTS cells are also stimulated by nicotine. The net result of these effects is increased GABAergic neurotransmission to pyramidal neurons, reduced dendritic calcium signaling and an increased threshold for spike-timing-dependent potentiation.

Somatic action potentials propagate deep into the dendritic tree and activate voltage-gated calcium channels in proximal and distal parts of dendrites, inducing substantial amounts of calcium influx in dendrites and dendritic spines (Koester and Sakmann, 1998; Stuart et al., 1997; Yuste and Denk, 1995). The control of dendritic action potential propagation, calcium signaling and spike-timing-dependent plasticity by inhibitory GABAergic transmission has been described in hippocampus (Meredith et al., 2003; Tsubokawa and Ross, 1996). Coincident activation of GABAergic inputs reduced dendritic action potential amplitude and the dendritic calcium signal associated with the action potential (Tsubokawa and Ross, 1996). Pairing of presynaptic activity with single postsynaptic action potentials becomes less effective at potentiating glutamatergic synapses with advancing developmental age. This results from increasing GABAergic inhibition during postnatal development, and can be overcome by pairing presynaptic activity with a burst of several postsynaptic action potentials (Meredith et al., 2003). Nicotinic AChR stimulation increases GABAergic transmission in hippocampus (Alkondon and Albuquerque, 2001; Alkondon and Albuquerque, 2004). Augmentation of GABAergic transmission by nAChR stimulation prevents long-term potentiation induced by 100 Hz stimulation for 1 sec (Ji et al., 2001). Which postsynaptic mechanisms are involved in this blockade of LTP is not known. In the PFC, we find that with the block of spike-timing-dependent potentiation of excitatory transmission dendritic calcium signaling is strongly reduced when GABAergic transmission is augmented by nicotine. Since transient increases in calcium concentration are fundamental to the induction of long-term potentiation (Koester and Sakmann, 1998; Magee and Johnston, 1997; Sjostrom and Nelson, 2002), also in PFC pyramidal neurons (Fig 7), this most likely explains why synaptic potentiation fails in the PFC in the presence of nicotine. Thus, stimulation of nAChRs on different types of neurons can change

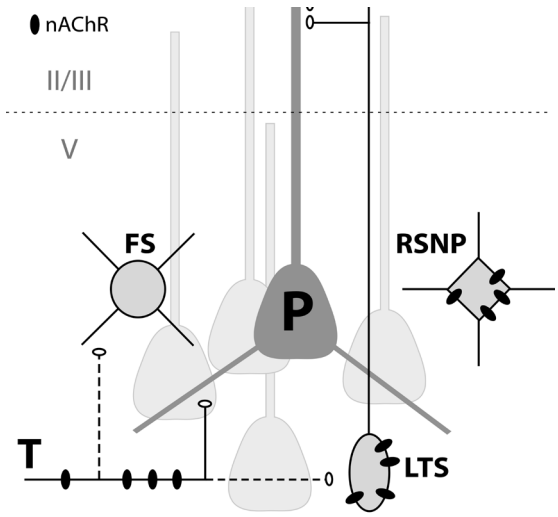


Figure 9. Schematic of the neuronal network of mouse layer V PFC depicting the distribution of nAChRs.
P Layer V pyramidal cell, FS Fast-spiking interneuron, RSNP Regular-spiking non-pyramidal neuron, LTS Low-threshold spiking neuron. LTS cells were drawn to synapse on the apical dendrites of pyramidal neurons, in line with the description of PFC Martinotti cells provided by Silberg and Markram (Silberberg and Markram, 2007)

the rules for induction of spike-timing-dependent plasticity in PFC, requiring stronger postsynaptic activity for potentiation to occur.
Nicotine reduced the amount of synaptic potentiation when presynaptic activity was paired to bursts of postsynaptic action potentials. The calcium transients induced by postsynaptic action potential bursts were similar in the presence and absence of nicotine (Fig 8). This suggests that nicotine could be affecting the synaptic plasticity machinery in PFC pyramidal neurons by other mechanisms in addition to reducing calcium signalling. It is well known that in other brain areas, nicotinic AChRs are also located on presynaptic neurons that are not glutamatergic or GABAergic, such as dopamine neurons (Wonnacott et al., 2000). It is unknown whether nAChRs are present on any of the monaminergic nuclei that project to the PFC. Dopamine is known to affect synaptic plasticity in PFC (Matsuda et al., 2006; Otani et al., 2003). Nicotinic modulation of dopamine and other monaminergic neurotransmission in the PFC could contribute to the observed modulation of STDP.
Modification of plasticity rules in cortical neuronal networks by nAChR activation may be a general phenomenon, extending beyond PFC and hippocampus. GABAergic interneurons and inhibitory synaptic transmission are affected by nAChR stimulation in many brain areas (Alkondon and Albuquerque, 2004; Mansvelder et al., 2006; Mansvelder et al., 2002; Metherate, 2004). Just as in hippocampus and PFC, pyramidal neurons in sensory cortical areas are not directly affected by nicotinic agonists, but different types of interneurons are strongly excited by postsynaptic nAChR activation (Alkondon and Albuquerque, 2004; Alkondon et al., 2000; Metherate, 2004; Xiang et al., 1998). Different types of nAChR subunits are involved, both α7 and α4β2-containing receptors. Many of these interneurons innervate pyramidal neurons, and therefore nAChR-mediated stimulation of interneurons would increase inhibition of pyramidal neurons. Just as we found in PFC pyramidal neurons, this could lead to reduced dendritic action potential propagation and reduced calcium signaling. As a result, stronger postsynaptic activity would be required to overcome this increased

dendritic inhibition to induce STDP.

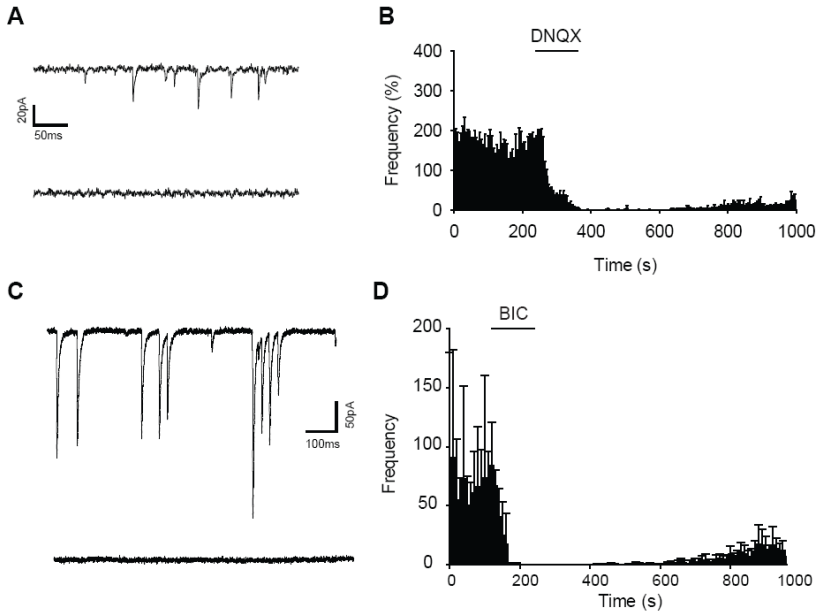
In vivo recordings show that glutamatergic projections between ventral hippocampus and prefrontal cortex can alter in strength during behavior (Laroche et al., 2000). Projections from the ventral hippocampus CA1 area enter the medial PFC through superficial layers I and II and project to all layers (Jay and Witter, 1991; Laroche et al., 2000). The extent to which synaptic plasticity of these inputs will be affected by nAChR stimulation will most likely depend on the dendritic location of the synapse. Since we found that nAChR stimulation can reduce dendritic calcium signaling associated with postsynaptic action potentials in apical dendrites of layer V pyramidal neurons, it is very likely that plasticity in glutamatergic synapses located distally are most affected by this reduction. At present, it is unknown whether glutamatergic fibers between hippocampus and prefrontal cortex express nAChRs. Glutamatergic fibers from thalamus that project to PFC layer V pyramidal neurons do express nAChRs and are directly stimulated by nAChR activation (Lambe et al., 2003). These receptors contain β2 subunits and are most likely not situated on the presynaptic glutamatergic terminals, since the effect of nicotine is mediated by an increase in action potential firing. Projections from the thalamus terminate in deep as well as superficial layers of the rodent medial PFC (Berendse and Groenewegen, 1991; Heidbreder and Groenewegen, 2003). Most likely, synaptic plasticity of thalamocortical terminals synapsing on the distal apical dendrite of layer V pyramidal neurons in superficial layers will suffer more from the nicotinic mechanisms we found to block STDP than the synapses that are located closer to the cell body. By reducing dendritic action potential propagation in apical dendrites, nicotine hampers communication between cell body and distal synapses in layer V pyramidal neurons. This potentially could strongly affect information processing in the neuronal network of the medial PFC as a whole, and will alter the output of the PFC.

The activation of distributed nAChRs provides the PFC neuronal network with a wide range of computational possibilities. Nicotine alters the rules for synaptic plasticity resulting from timed presynaptic and postsynaptic activity by increasing the threshold. Thereby the function of the medial PFC network will most likely change in the presence of nicotine. Increased activity in pyramidal neurons at least partially restores the conditions for STDP to occur. The presence of nicotine and increased threshold for STDP could reduce cognitive performance in healthy naïve rodents (Day et al., 2006). Alternatively, since PFC neuronal activity could be increased during PFC-based cognitive behavior, nicotine may provide conditions under which signal to noise ratio in PFC information processing is enhanced, thereby improving cognitive performance (Day et al., 2006; Mirza and Stolerman, 1998).

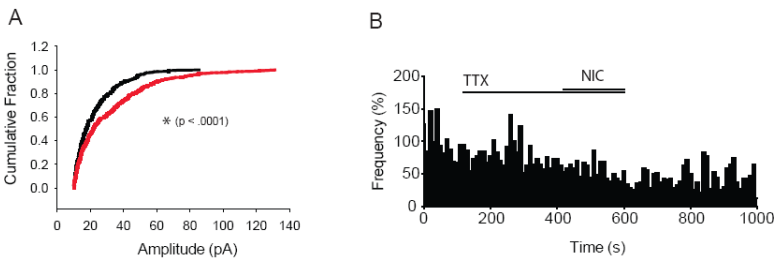
Acknowledgements

We thank Tessa Lodder, Hans Lodder and Jaap Timmerman for excellent technical support. This work was supported by grants from the Faculty of Earth and Life Sciences

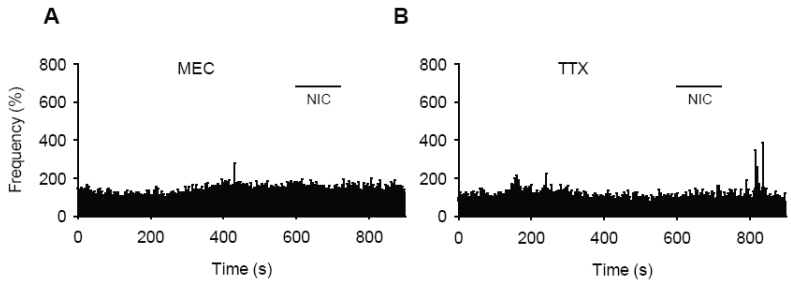
and Center of Medical Systems Biology (ABS), the Dutch Medical Research Council (ZonMW, 911-03-014 to HDM and ABB; and ZonMW 016-076-360 to HDM), and the Royal Netherlands Academy of Arts and Sciences (KNAW, to HDM). Both CNCR departments are financially supported by NeuroBsik (www.neurobsik.nl)



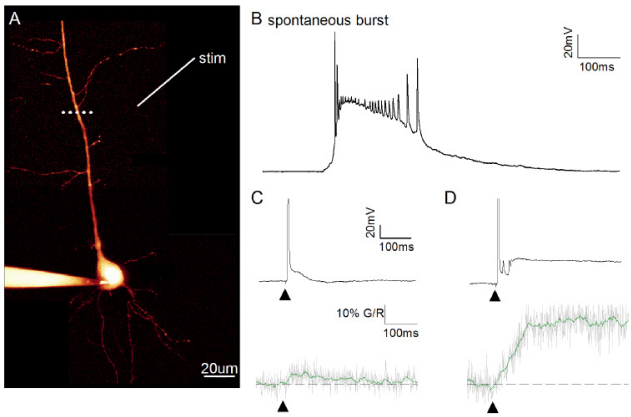
Supplementary Figure 1.
A. upper trace: Example trace with spontaneous excitatory postsynaptic currents (EPSCs) recorded from layer 5 pyramidal neurons. Inhibitory postsynaptic currents (IPSCs) are not visible since the membrane potential was clamped at the reversal potential for chloride. lower trace: Example trace in the presence of the AMPA receptor blocker DNQX (10 μM). Note that no EPSCs are present any longer. B. Average frequency histogram of EPSCs (n=4). C. upper trace: Example trace with spontaneous IPSCs recorded from layer 5 pyramidal neurons. EPSCs were blocked by DNQX (10 μM). lower trace: Example trace in the presence of bicuculline (10 μM). D. Average frequency histogram of IPSCs (n=3).



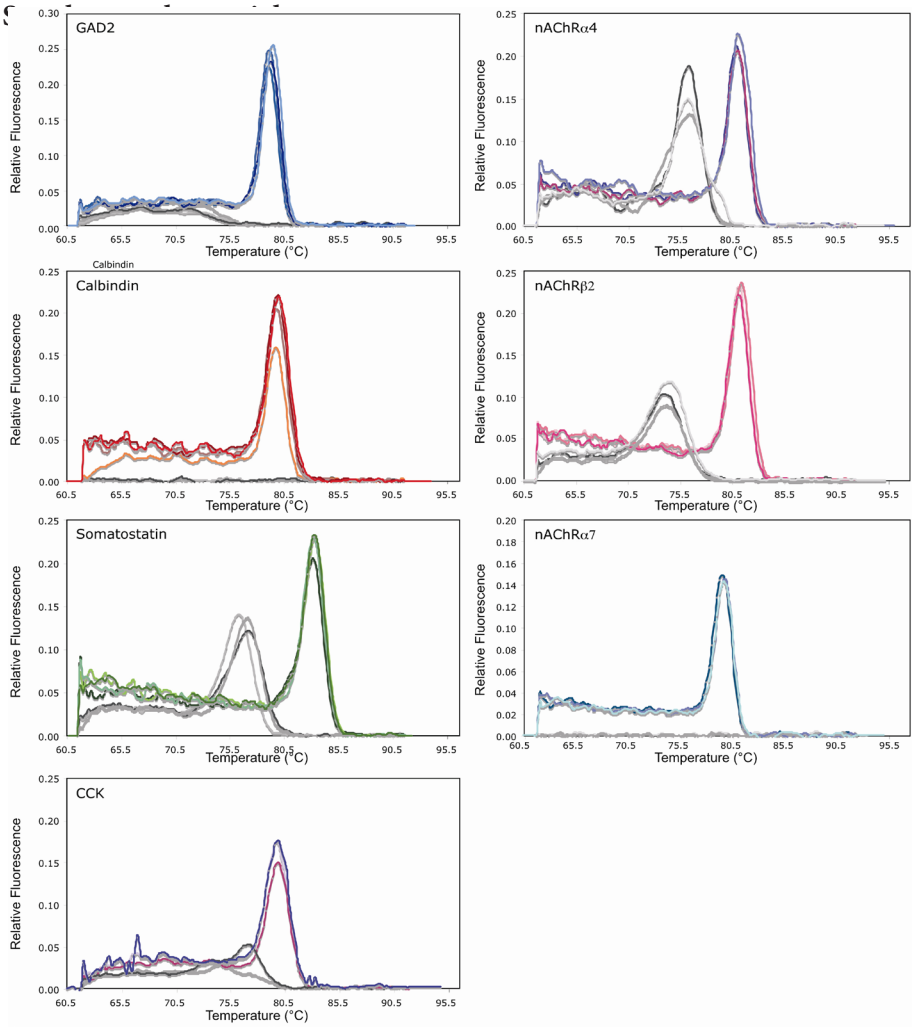
Supplementary Figure 2
A Cumulative IPSC amplitude distributions recorded from four pyramidal neurons in the presence of MLA before (black) and after (red) application of nicotine (10 μM). Nicotine shifted the amplitude distribution to larger amplitudes. B. TTX (1 μM) prevented the effect of nicotine on the frequency of IPSCs



Supplementary Figure 3.
A. Average histogram of spontaneous EPSCs recorded from FS interneurons in the presence of mecamylamine (1 μM, n=10). Nicotine (10 μM) no longer increases the frequency of EPSCs. B. The effect of nicotine on EPSC frequency in FS interneurons is also prevented by TTX (1 μM, n=6).



Supplementary Figure 4.
Spontaneous bursting observed in layer 5 pyramidal cells following bath application of gabazine and nicotine. A. Layer 5 pyramidal filled with Alexa 594 (40 μM) and Fluo4 (100 μM). Dotted line indicates position of linescan across the dendrite. S – stimulation electrode. B. Spontaneous burst observed in another layer 5 PFC pyramidal cell following bath application of 1μM Gabazine. C. Trace showing EPSP-AP stimulation (upper panel) and corresponding dendritic calcium transient (lower panel) recorded from cell in A. D. In some excluded cases, identical EPSP-AP stimulation paradigms caused prolonged somatic depolarization (upper panel) and resulted in large prolonged calcium transients (lower panel).



Supplementary Figure 5.
Dissociation curves were performed for each reaction after 45 cycles of the re-amplification (real-time PCR) to check the specificity of the formed product. Examples are given for the positive control and positive cells (colored lines), as well as for the negative control (dark gray) and negative cells (gray lines). Note that in some cases, the negative control yielded a product only after re-amplification. Negative cells could be discriminated based on the melting temperature of the product, which is represented by peak of the curve. Relative fluorescence (i.e. the derivative of the fluorescence) and temperature are indicated.

Layer-specific interference with cholinergic signaling in the prefrontal cortex by smoking concentrations of nicotine

Poorthuis RB, Bloem B, Verhoog MB, Mansvelder HD.

Chapter 5

Published in Journal of Neuroscience 2013 Mar 13;33(11): 443-53

Abstract

Adolescence is a period in which the developing prefrontal cortex (PFC) is sensitive to maladaptive changes when exposed to nicotine. Nicotine affects PFC function and repeated exposure to nicotine during adolescence impairs attention performance and impulse control during adulthood. Nicotine concentrations experienced by smokers are known to desensitize nicotinic acetylcholine receptors (nAChRs), but the impact thereof on PFC circuits is poorly understood. Here, we investigated how smoking concentrations of nicotine (100 – 300 nM) interfere with cholinergic signaling in the mouse PFC. nAChR desensitization depends on subunit composition. Since nAChR subunits are differentially expressed across layers of the PFC neuronal network, we hypothesized that cholinergic signaling through nAChRs across layers would suffer differentially from exposure to nicotine. Throughout the PFC, nicotine strongly desensitized responses to ACh in neurons expressing β2* nAChRs, whereas ACh responses mediated by α7 nAChRs were not hampered. The amount of desensitization of β2* nAChR currents depended on neuron type and cortical layer. β2*-mediated responses of interneurons in LII-III and LVI completely desensitized, while cholinergic responses in LV interneurons and LVI pyramidal cells showed less desensitization. This discrepancy depended on α5 subunit expression. Two-photon imaging of neuronal population activity showed that prolonged exposure to nicotine limited cholinergic signaling through β2* nAChRs to deep PFC layers where α5 subunits were expressed. Together, our results demonstrate a layer-dependent decrease in cholinergic activation of the PFC through nAChRs by nicotine. These mechanisms may be one of the first steps leading up to the pathophysiological changes associated with nicotine exposure during adolescence.

Introduction

Despite negative health consequences, tobacco smoking remains a persistent drug addiction worldwide (WHO, 2012). First experiences with cigarette smoking often take place during adolescence (Escobedo et al., 1993; Currie et al., 2008). The prefrontal cortex (PFC), which is involved in higher order processes such as attention, impulse control and working memory (Groenewegen and Uylings, 2000; Miller, 2000), continues to develop during this period (Gogtay et al., 2004). As a consequence, exposure to nicotine during adolescence compromises normal PFC development (Counotte et al., 2011b; Goriounova and Mansvelder, 2012a). Repeated exposure to nicotine transiently increases nicotinic acetylcholine receptor subunit (nAChR) expression and GABAergic synaptic transmission in the PFC (Counotte et al., 2012). Secondary to this, a decrease of mGluR protein persists into adulthood and causes altered synaptic learning rules and attention behavior (Counotte et al., 2011a; Goriounova and Mansvelder, 2012b). Despite these insights into long-term changes of PFC function after nicotine exposure, it is still unclear what the initial mechanisms are by which nicotine alters cortical processing at the neuronal network level.

Rapid, phasic cholinergic signaling within the PFC is crucial for attention behavior (Parikh et al., 2007; Sarter et al., 2009) and disturbances in cholinergic signaling impair attention (Turchi and Sarter, 1997; Newman and McGaughy, 2008). nAChRs are fast ionotropic receptors and their activation kinetics suggests that they are efficiently activated by rapid increases in acetylcholine. Attention performance depends on functional nAChRs in the medial PFC (Guillem et al., 2011). Nicotinic receptors activate the PFC in a layer specific manner (Poorthuis et al., 2013). In superficial layers only interneurons are activated whereas in deeper layers pyramidal neurons and interneurons are modulated by nAChRs. Short exposure to nicotine alters synaptic transmission and rules for plasticity induction (Couey et al., 2007). However, during smoking, blood levels of nicotine in smokers remain elevated and reach peak levels of 300 to 600 nM (Matta et al., 2007). These concentrations desensitize neuronal nAChRs (Mansvelder et al., 2002; Wooltorton et al., 2003; Grady et al., 2012). It is not known whether desensitization plays an important role in the PFC. The presence of α5 subunits protects β2-containing receptors in layer VI pyramidal neurons from desensitization (Bailey et al., 2010). In the PFC, α5 nAChR subunits are highly expressed (Counotte et al., 2012), but α5 subunit expression has been reported to be much lower in superficial cortical layers (Wada et al., 1990; Winzer-Serhan and Leslie, 2005). It is unknown how nicotine affects cholinergic transmission in these layers and whether α7 nAChR activation is affected by nicotine.

We tested the hypothesis that nicotine interferes with cholinergic activation of the PFC network through nAChRs and that this effect is more prominent in superficial layers. Using electrophysiological recordings and two-photon network imaging we find that desensitization in response to nicotine is cell type and layer specific and that

this can be explained by the presence of the nAChR α5 subunit. As a consequence, in the presence of nicotine cholinergic signaling through β2* nAChRs is restricted to layer VI.

Materials and methods

Prefrontal cortical slice preparation

Prefrontal coronal cortical slices (300μM) were prepared from P14-P21 and P34-43 C57BL/6 mice or α5 wildtype and α5 null littermates P34-43 of either sex, in accordance with institutional and Dutch license procedures. Following rapid decapitation, the brain was removed from the skull in ice-cold artificial cerebrospinal fluid containing 125 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 3 mM MgSO4, 1 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose (~300 mOsm). After removal of the cerebellum the brain was glued on this plane to create a coronal orientation for cutting slices. Slices were then transferred into holding chambers containing aCSF 125 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 1 mM MgSO4, 2 mM CaCl2, 26 mM NaHCO3, and 10 mM glucose (~300 mOsm) and bubbled with carbogen gas (95% O2 / 5% CO2) to recover for at least an hour.

Electrophysiology

Slices were transferred to the recording chamber and perfused with standard aCSF (2-3 ml/min). All experiments were performed at 31-34° C. Cells were visualized using differential interference contrast microscopy. Recordings were made using Multiclamp 700B amplifiers (Axon Instruments, CA), sampled at a frequency of 20 kHz, digitized by the pClamp software (Axon), and later analyzed off-line. Patch pipettes (3-5 MOhms) were pulled from standard-wall borosilicate capillaries and were filled with intracellular solution: 140mM K-gluconate, 1mM KCl, 10mM HEPES, 4mM K-phosphocreatine, 4 mM ATP-Mg, and 0.4 mM GTP (pH 7.2–7.3, pH adjusted to 7.3 with KOH) (290–300 mOsm) and biocytin (4mg/ml) (used for EPSC and puff application experiments, reversal potential chloride ~-127 mV, hence IPSCs in this case are detected as outward currents). Action potential profiles of cells were made using hyperpolarizing and depolarizing current steps. For IPSC experiments a modified intracellular solution was used with a high chloride concentration (70mM K-gluconate and 70 mM KCl) to augment GABAergic currents (reversal potential for chloride is ~-16 mV, hence GABA currents are detected as inward currents). All IPSC experiments were done in the presence of DNQX (10μM). All experiments recording inhibitory or excitatory postsynaptic currents were done in the presence of atropine (200 nM), to prevent muscarinic receptor stimulation. For network experiments acetylcholine (1 mM) was bath applied. Nicotine (Sigma, 300 nM or 3000 nM) was bath applied in all experiments.

Nicotinic receptor currents on interneurons and pyramidal neurons were tested

by pressure ejection of acetylcholine (Sigma, 1mM) for 100 ms using a Picospritzer III (General valve corporation, Fairfield, NJ) from a glass electrode with a tip opening of ~ 1 µm. The puffer pipette was located ~ 20 µm from the soma and placed in perpendicular direction with respect to the pial surface. The presence of atropine (200 nM) prevented stimulation of muscarinic receptors and during all experiments and DNQX (10 µM) and bicuculline (1 µM) were used to block synaptic transmission. Nicotine (Sigma, 100 nm and 300nM) was bath applied in all experiments.

Analysis and statistics for electrophysiological experiments

Frequency of excitatory or inhibitory postsynaptic currents (PSC's) was analyzed using MiniAnalysis (Synaptosoft, Inc). Local pressure application experiments were analysed using custom made software for Matlab (Mathworks). The effect of nicotine on cholinergic signaling was determined by calculating the charge of ACh-induced currents before during and after exposure to nicotine. In case cells showed a mixed α7 / β2 mediated nAChR current, the charge of the β2 current was calculated after the α7 current ended (~300 ms). The different receptor currents were well distinguishable by the different rise times of the two components and the full α7 component remained after desensitization. In addition, the β2 currents are more than ten times longer than α7 currents (~3-10 s), hence taken out the α7 had little influence on determining the charge of the β2 receptor. In Fig 1A3 and 1B3 only the charge of the β2 component was plotted, while the α7 component was not plotted. To test for frequency differences in PSC's we used a Student's t-test. To test for effects of pharmacology or genotype effects on nAChR charge induced by puff application of ACh a Student's t-test was used. Statistical tests for stable baseline currents were done on the raw data. Statistical tests for effects of desensitization were done on normalized data and done by comparing the last data point before nicotine application with the first data point after 10 minutes of nicotine. In all desensitization experiments analysis was done on the charge of the nAChR currents. Significant results were obtained with a p-value <0.05. p-values between 0.05 and 0.01 are shown as <0.05. p-values between 0.01 and 0.001 are shown as p<0.01 and p-values lower than 0.001 are shown as p<0.001.

Two photon calcium imaging

Loading

Slices were made as described before, but in an alternative slicing solution (27mM NaHCO3, 1.5mM NaH2PO4, 222mM sucrose, 2.6mM KCl, 0.5mM CaCl2, 3mM MgSO4). Hereafter, slices were incubated in regular aCSF at 35oC for 20min and in room temperature for another 40min. For bulk loading, a modified protocol based on (Trevelyan et al., 2006) was used. Briefly, slices were first preincubated at 37oC for 5 min in 3ml aCSF containing 8µl Cremophor EL

solution (0.5% Cremophor EL in DMSO). After this, 1µl Fura-2AM solution (25µg Fura-2AM in 4.5µl DMSO and 0.5µl pluronic acid) was pipetted on top of each slice.

Then the slices were left for incubation for 35-40min after which they were put back in the slice chamber with aCSF at room temperature for at least 45min. Imaging experiments were performed in aCSF (perfusion speed 2.5ml/min), continuously bubbled with 95% O2/ 5% CO2, at 32oC. Imaging was performed using a multibeam two-photon laser scanning microscope system (Trimscope, Lavision BioTec) coupled to a Ti:Sapphire laser (Chameleon, Coherent, excitation at 820nm) and a CCD camera (C9100 Hamamatsu). The objective used had a 20X magnification and a 0.95 numerical aperture. The imaged plane was always in the same orientation with respect to the pia and the distance between them was determined for later analysis. The imaged area was 400X400 μm (pixel size of 0.8μm, binning 2X2) and the imaging frequency was 9Hz.

Experimental protocol

Baseline activity was imaged during a 4 minute period. After this, nicotine (300 nM) was applied for 10 minutes. During the first 4 minutes of nicotine perfusion, the activity in the slice was imaged. Then ACh (1 mM) and nicotine (300 nM) were applied for 2 minutes after which the drugs were washed out (8 minutes). During these periods imaging took place.

Analysis

Analysis was done using custom made software for Matlab (Mathworks). This program detected cell contours and extracted the fluorescence within these contours as a function of time. After this, cell activity was determined per minute in a blind fashion. Cells were divided in three depth groups, corresponding to the measured thicknesses of the three layers in the PFC. Neurons that were between 100 and 300 μm, between 300 and 550 μm and between 550 and 800 μm were considered to be part of respectively layer II/III, V and VI. For determining the activity in the different drug conditions, the percentage of neurons showing at least one calcium event was calculated per slice per minute. If slices included multiple layers, then the slice was split up into two new slices containing just one layer. Effects of drugs, layer and condition were tested using repeated measures ANOVA, followed by Fisher's LSD posthoc tests. After this, for direct comparison of the activations in the different celltypes in the different conditions, it was determined per neuron whether the activity after ACh application was higher, lower or equal to the amount of calcium events in the minute before ACh application. Chi square tests were performed to test if this statistic was different for the multiple layers, condition and neuron types. In addition, binomial tests were used to determine the significance of the activation for every combination.

Determination of cell identity

High resolution z-stacks were made to optimize the possibilities for identification (voxel size: 0.4 X 0.4 X 0.5 μm). For the majority of neurons, proximal dendrites showed

strong fluorescence. Cells were only taken into account if dendritic fluorescence was sufficient and cells could be identified as interneurons or pyramidal neurons according to the following criteria: 1. the presence of a clear apical dendrite, 2. a pyramidal shaped cell body for pyramidal neurons. 3. a clear non-pyramidal cell body morphology. 4. bipolar or multipolar dendrite morphologies for the interneurons. Criteria 1 and 2 classified the neuron as pyramidal. Criteria 3 and 4 classified a neuron as interneuron. If the dendrites were not visible in the z-stack, the neurons were not categorized. Identification of cells was done in a blind manner, i.e. the experimenter was unaware of whether neurons were activated by nicotine receptor stimulation or not, excluding the possibility of a bias. After morphological identification, data were compared to electrophysiological experiments. If neurons could not be unequivocally identified, they were excluded from statistics on cell type specific activation.

Results

Desensitization of LII-III β2-nAChR current responses by smoking concentrations of nicotine*

To test the hypothesis that nAChR currents desensitize more strongly in PFC LII-III than in LVI, we first targeted layer II-III non-fast-spiking (NFS) interneurons (Figure 1A2), the only cell type in this PFC layer that expresses β2-containing nAChRs (Figure 1A1) (Poorthuis et al., 2013). ACh-induced β2* nAChR-mediated currents had slow rise and decay times, were blocked by dihydro-β-erythriodine (DHβE) and were absent in β2 null mice (Figure 1A3) (Poorthuis et al., 2013). nAChR currents were induced by pressure application of ACh (1 mM, 100 ms) at two minute intervals (Figure 1A4). These applications induced repeatable postsynaptic currents that were stable over time (Figure 1B1, the third versus the first response, 100% vs. 98.4 ± 14 %, Student's t-test, $p=0.49$. We then tested the effect of a ten minute nicotine application of 300 nM, which resembles arterial blood concentration profiles during cigarette smoking (Matta et al., 2007), on these ACh-induced currents. After ten minutes of nicotine application, responses to ACh were strongly reduced on LII-III NFS interneurons (Figure 1B1 and B3, n=5, 17.4 ± 0.06 % remaining response, $p<0.01$). The reduction of ACh-induced currents remained after nicotine was washed-out from the bath for up to 45 minutes (Figure 1B1, n=4; @ 15 min 45.2 ± 10 % remaining response, $p<0.01$; @ 30 min 64.7 ± 9.0 % remaining response, $p<0.05$; @ 45 min 83.4 ± 11 % remaining response, $p=0.11$; @ 60 min 78 ± 6 % remaining response, $p=0.40$). This suggests that β2* nAChRs expressed by PFC LII-III NFS cells were desensitized by exposure to smoking concentrations of nicotine.

Adolescence (P34-P43) is a period in which rodents are in particular vulnerable for the effects of nicotine on PFC-dependent cognitive functioning (Counotte et al., 2011a). Nicotinic AChR subunit expression changes during development and may therefore alter the sensitivity of receptors for nicotine and desensitization. To test

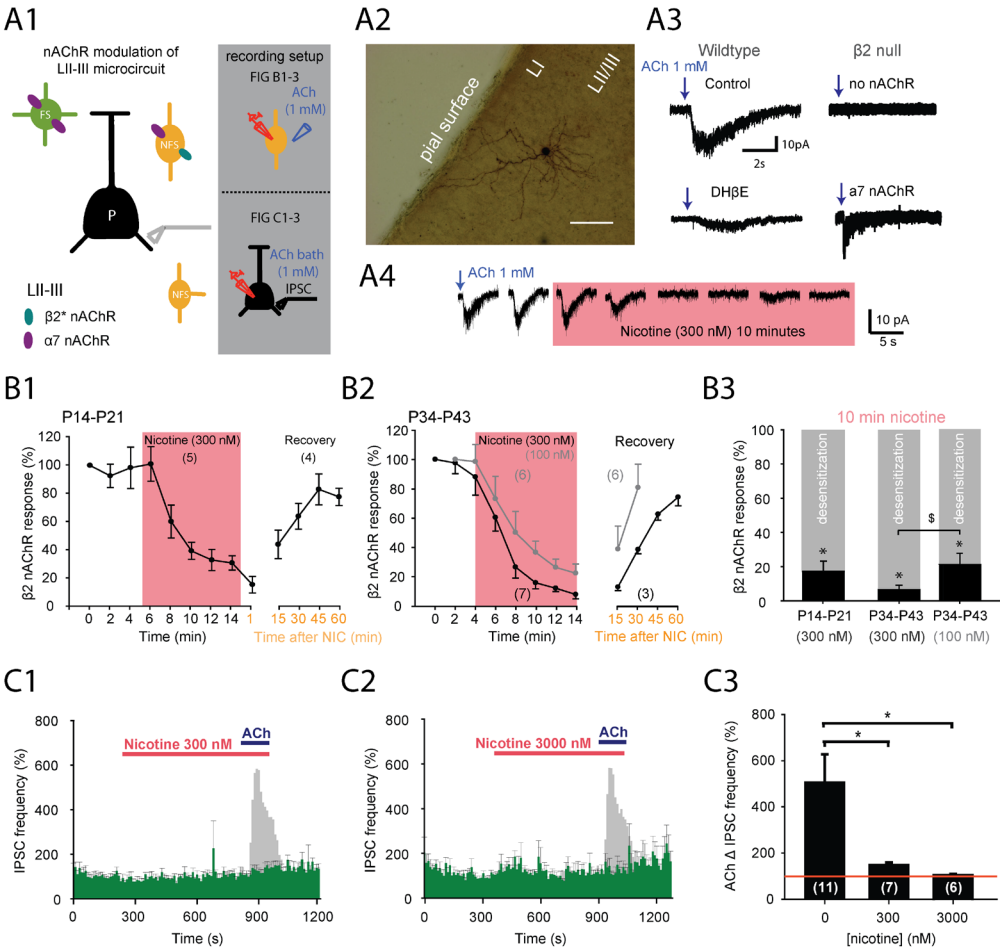


Figure 1. Desensitization of LII-III β2*-nAChR responses by smoking concentrations of nicotine.
(A1) Schematic showing nAChR receptor distribution in PFC LII-III microcircuitry. FS = Fast-Spiking interneuron, NFS= Non-fast-Spiking interneuron, P= Pyramidal neurons. Grey synapse = glutamatergic input and black synapse = inhibitory input. β2* nAChRs and α7 nAChRs are indicated with turquoise and purple colored ovals. Right panel shows the recording configuration used to test for desensitizing effects of nicotine on LII-III β2*-nAChR responses.
(A2) Morphological staining of a LII-III NFS interneuron in the adolescent PFC. Scale bar = 100 µm.
(A3) β2* nAChRs on NFS interneurons are characterized by slow rise and decay kinetics and are blocked by DHβE (wildtype example traces). In β2 null mice these current are absent and only short-lasting currents with a fast rise-time characteristic of α7 nAChRs remain (right example traces, see Poorthuis et al., 2013).
(A4) Example trace showing β2* nAChR currents in LII-III of the adolescent PFC evoked by puff application of ACh (1 mM) every two minutes. Low concentrations of nicotine (300 nM, 10 min, pink shading) completely abolish β2* nAChR currents in LII-III.
(B1) Average surface area of current responses of juvenile LII-III NFS interneurons to local ACh (1 mM) application during bath exposure to nicotine (300 nM, 10 min). Current charge remains reduced when nicotine is washed out of the bath for up to 45 minutes.

(B2) Same as in B1, but now for adolescent NFS interneurons. In grey the effect of exposure to 100 nM of nicotine is shown. Note that the desensitisation rate is slower and recovery from desensitization quicker.
(B3) Summary histogram quantifying the desensitizing effect of a ten minute nicotine (300 nM) application on the current charge of β2* nAChRs in juvenile (n=6, Student's t-test, p<0.01) and adolescent LII-III NFS interneurons (n=7, p<0.01). The degree of desensitization was not different between the age groups (p=0.15). The right bar shows that 100nM nicotine also strongly interfered with ACh-induced β2* mediated currents in adolescent LII-III NFS neurons (n=6, p<0.01), but less compared to 300 nM nicotine (p=0.03, indicated with $)
(C1) Histogram showing that nicotine abolished the effect of ACh on inhibitory transmission to layer II-III pyramidal neurons. Response without nicotine is shown in grey.
(C2) Same experiment as in A1, but now for 3000 nM nicotine.
(C3) Summary showing the effect of nicotine on ACh induced increase of inhibitory transmission to layer II-III pyramidal neurons. Nicotine completely abolished cholinergic control over inhibitory transmission (300 nM, n=7, p=0.03; 3000 nM, n=6, p=0.02). All statistical tests for figure 1-6 used Student's t-test. * denotes significance within test group, $ denotes significance between test groups.

whether nAChR-mediated currents in the adolescent PFC similarly desensitize, we performed the same experiment in mice at this developmental period. Acetylcholine application induced stable currents (Figure 1B2, second versus third response, 100% vs. 87.9 ± 12.5, p=0.96). Nicotine application abolished ACh-induced β2* mediated currents in adolescent LII-III NFS neurons (Figure 1B2 and B3, n=7, 6.7 ± 2.5 % remaining after ten minutes of nicotine, p<0.01). Similar to the ACh responses in juvenile neurons, β2*-nAChR-mediated responses were reduced for a prolonged period of time in adolescent neurons (Figure 1B2; n=3, @ 15 min 10.9 ± 2.6 % remaining response, p=0.02; @ 30 min 37.1 ± 2.9 % remaining response, p=0.06; @ 45 min 61.8 ± 4.2% remaining response, p=0.28; 60 min 73.7 ± 6.0 % remaining response, p=0.99), suggesting that also in adolescent PFC neurons β2*-nAChRs strongly desensitize. Two out of seven recorded cells contained a mixed β2*- and α7*-nAChR-mediated response. In these cells, the α7 component was not desensitized by nicotine (data not shown). We also tested whether a lower nicotine concentration, as observed in smokers between cigarettes in the afternoon (Matta et al. 2007), would have a desensitizing effect on nicotinic receptor currents. Application of 100nM nicotine strongly reduced ACh-induced β2* mediated currents in adolescent LII-III NFS neurons (Figure 1B2 and B3, n=5, 21.4 ± 6.3 % remaining after ten minutes of nicotine, p<0.01), but the reduction was less compared to 300 nM nicotine (Figure 1B3, p=0.03).

Activation of β2* nAChRs enhances GABAergic signaling onto LII-III pyramidal neurons in the PFC (Couey et al., 2007; Poorthuis et al., 2009, 2013). We tested whether nicotine (300 nM) interferes with cholinergic modulation of inhibitory postsynaptic currents (IPSCs) received by pyramidal neurons by applying nicotine for ten minutes followed by co-application of nicotine and ACh (1 mM). In the absence of nicotine, ACh dramatically increases the frequency of IPSCs in layer II-III pyramidal neurons (Figure 1C3; n=10, 505.3 % ± 148.2, p<0.01). After exposure to nicotine, ACh hardly increased IPSC frequency anymore (Figure 1C1-3; 300 nM nicotine, n=7, 122.7 %

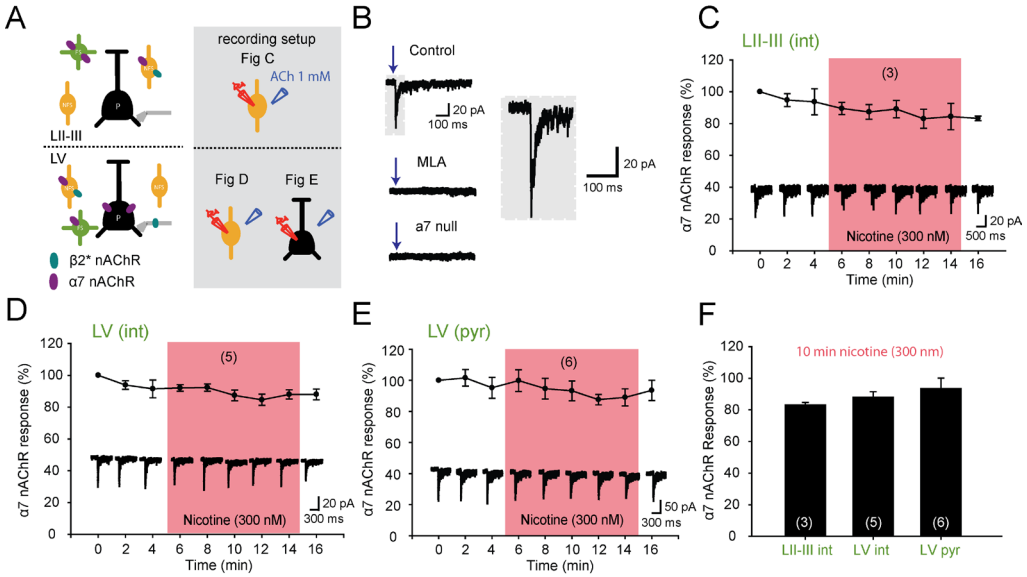


Figure 2. Smoking concentrations of nicotine do not affect α7 nAChR currents.
(A) Left panel: nAChR modulation of PFC microcircuitry in LII-III and LV. β2* nAChRs and α7 nAChRs are indicated with turquoise and purple colored ovals. Right panel in grey shading shows the recording setup for the different experiments.
(B) Characteristics of α7 nAChRs. Currents show rapid activation and desensitization kinetics and are blocked by methyllycaconitine (MLA) and absent in α7 null mice (see Poorthuis et al., 2013). In grey shading a magnification of an α7 current is shown.
(C) Effect of nicotine (pink shading, 300 nM, 10 min) on α7 nAChR current responses induced by ACh (1 mM) application on juvenile LII-III interneurons. Nicotine does not interfere with a7 nAChR activation in the PFC.
(D) Same as in (C) but now for interneurons in layer V.
(E) Same as in (C) but now for layer V pyramidal neurons.
(F) Summary bar graph showing the effect of smoking concentrations of nicotine on α7 (300 nM, 10 min) nAChR currents. Nicotine exposure did not desensitize α7 nAChR currents throughout the PFC (Student's t-test, p=0.31, p=0.69 and p=0.25 for respectively LII-III interneurons, LV interneurons and LV pyramidal neurons).

± 11.3, p=0.06; 3000 nM nicotine, n=6, 104.8 ± 6%, p=0.09; ACh-control vs. ACh-nicotine (300 nM), p=0.03). Together, these data suggest that smoking concentrations of nicotine desensitize β2* nAChRs in LII/III. Thereby, nicotine interferes with cholinergic control through nAChRs over inhibitory circuits in superficial layers of the PFC.

Smoking concentrations of nicotine do not affect cholinergic signaling through α7 nAChRs

In other brain areas, nAChRs containing α7 subunits suffer less from desensitization by low concentrations of nicotine than β2-containing nAChRs (Mansvelder et al., 2002; Wooltorton et al., 2003). In the PFC, α7 nAChRs are expressed by LII-III and LV fast-

spiking and non-fast-spiking interneurons, as well as by LV pyramidal neurons (Figure 2A, Poorthuis et al., 2013). We hypothesized that in the PFC cholinergic signaling through α7 nAChRs is not influenced by concentrations of nicotine experienced by smokers. We targeted interneurons in LII-III positive for α7 nAChRs. α7 nAChR-mediated currents had a fast rise and decay time, were blocked by methyllycaconetine (MLA) and were absent in α7-null mice (Figure 2B, Poorthuis et al., 2013). Similar to β2* nAChRs, repeated ACh-induced currents mediated by α7 nAChRs were stable and showed a constant amount of charge (Figure 2C, third versus first response, 100% versus 94 ± 8 %, p=0.3). Subsequent exposure of the receptors to 300 nM of nicotine for ten minutes did not significantly alter ACh-induced currents (Figure 2C and 2F, 83.0 ± 1.4 % remaining after ten minutes of nicotine, p=0.31). A similar result was obtained for layer V interneurons (Figure 2D and 2F, 88.0 ± 3.4 % remaining after ten minutes of nicotine, p=0.69) as well as layer V pyramidal neurons (Figure 2E and 2F, 93.5 ± 6.6 % remaining after ten minutes of nicotine, p=0.25). Hence, these data show that nicotine concentrations seen in smokers during cigarette smoking do not hamper cholinergic stimulation of α7 nAChRs in the PFC.

Partial interference of nicotine with β2 nAChR-mediated cholinergic responses in LV*

In layer V of the PFC, β2* nAChRs are found on glutamatergic inputs and non-fast-spiking interneurons. Stimulating the latter increases inhibitory inputs to pyramidal neurons (Figure 3A1, Poorthuis et al., 2013). Non-fast spiking interneurons in juvenile mice were targeted and tested for the effect of nicotine on β2* nAChR-mediated cholinergic responses. A ten minute application of nicotine (300 nM) strongly reduced β2* nAChR-mediated responses (Figure 3A2 and A3, 30.6% ± 4.0 remaining charge, n=9, p< 0.01). However, compared to the reduction in ACh-induced current by nicotine in LII-III NFS neurons, the reduction in NFS neurons in LV was less complete and a substantial ACh-induced current remained (Figure 3A2-3). Thus, β2-containing nAChRs expressed by LV NFS neurons desensitized to a lesser extent than β2-containing nAChRs expressed by LII-III NFS neurons (Figure 3A3, p<0.05).

Spontaneous IPSC's received by LV pyramidal neurons were strongly enhanced by ACh application (Figure 3B1 and B3, n=16, 351 ± 41%, p<0.01). After nicotine application, ACh still increased IPSC frequency (Figure 3B1 and B3, n=10, 171 ± 22%, p=0.03), but less than in control conditions (p<0.01). A high dose of nicotine (3000 nM) abolished ACh modulation through nAChRs of IPSCs (Figure 3B2 and B3, n=8, 112 ± 5%, p=0.08). Thus, in line with the results on LV NFS neurons, nicotine only partially interfered with cholinergic modulation through β2* nAChRs of IPSCs received by LV pyramidal neurons.

Activation of β2* nAChRs strongly enhances glutamate release from thalamic projections to PFC LV pyramidal neurons (Lambe et al., 2003). Nicotine (300 nM) partially reduced the ACh-induced increase in frequency of spontaneous excitatory

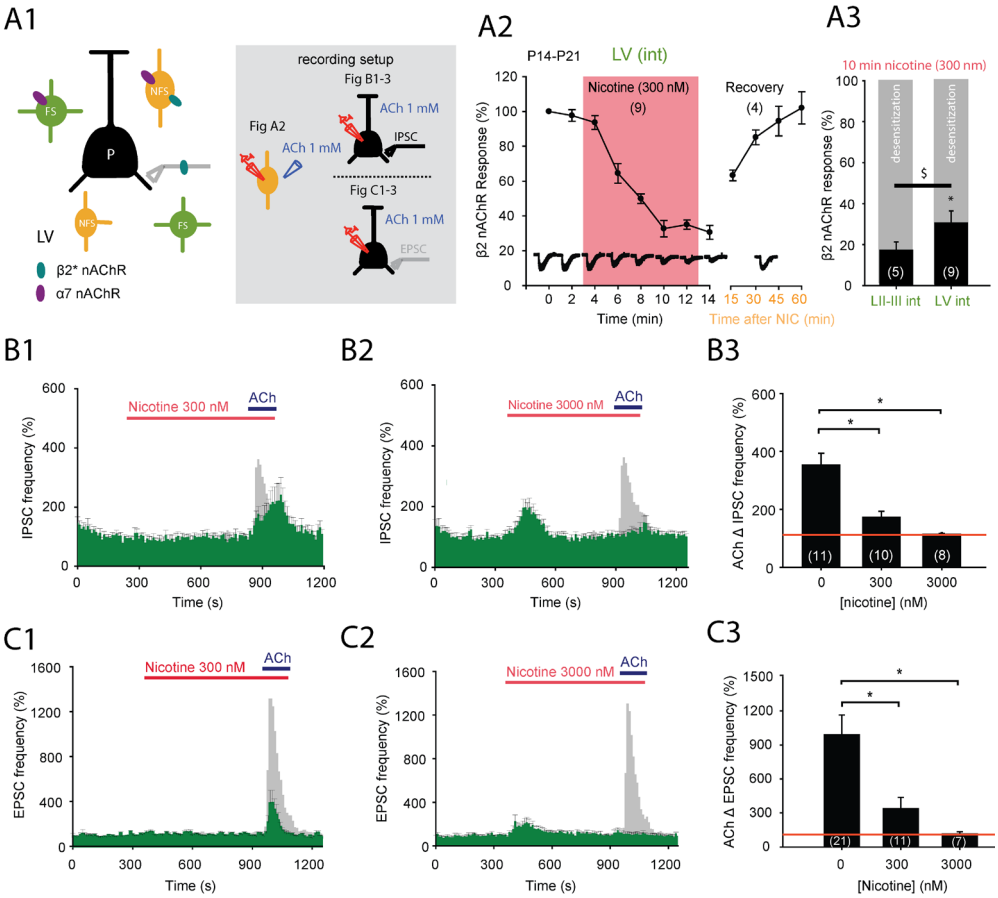


Figure 3. Partial interference of nicotine with β2* nAChR mediated currents in LV.
(A1) Microcircuitry showing nAChR distribution in layer V of the PFC. On the right, in grey shading, the recording setup for figures 5A-C is shown.
(A2) Average current responses of juvenile LV NFS interneurons to local ACh (1 mM) application during bath exposure to nicotine (300 nM, 10 min, pink shading). ACh induced currents are not completely abolished after ten minutes. Currents remain smaller for up to 45 minutes when nicotine is washed out of the bath.
(A3) Summary histogram showing the desensitizing effect of nicotine on ACh induced β2* nAChR responses. Nicotine significantly interferes with β2* nAChR currents in LV NFS-interneurons ($p<0.01$, indicated with *), but the desensitization is less compared to LII-III (Student's t-test, $p<0.05$, indicated with $)
(B1) Histogram showing nicotine only partially interferes with the effect of ACh on inhibitory transmission to layer V pyramidal neurons. Response without nicotine is shown in grey.
(B2) Same experiment as in A1, but for 3000 nM nicotine.
(B3) Summary showing the effect of nicotine on the ACh-induced increase of inhibitory transmission to layer V pyramidal neurons (300 nM, $p<0.01$; 3000 nM, $p<0.01$).
(C1-3) Same experiment as in B1-3, but now for spontaneous excitatory transmission (300 nM, $p<0.01$; 3000 nM, $p<0.01$). Response without nicotine is shown in grey.

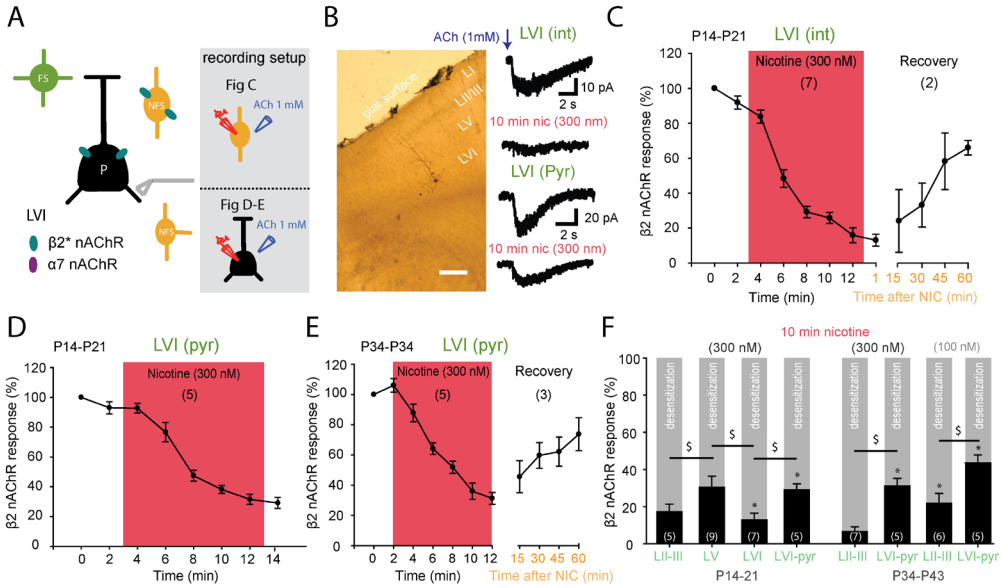


Figure 4. Differential desensitization of β2*-mediated nAChR currents in layer VI.
(A) nAChR modulation of layer VI microcircuitry. On the right, in grey shading, the recording setup for the different experiments is displayed.
(B) Morphological staining of an adolescent LVI pyramidal neuron. Scale bar = 250 μm. On the right example traces are shown of acetylcholine induced β2* nAChRs of layer VI neurons before and after exposure to nicotine (300 nM, 10 min).
(C) Average current responses of β2* nAChR currents during baseline and during washin of nicotine (300 nM, 10 min). Nicotine strongly reduces current responses of LVI interneurons.
(D) Same as in (C) but now for LVI pyramidal neurons
(E) Same as in (D) but now for adolescent mice
(F) Summary bar graph showing desensitization of β2* nAChRs in the PFC. nAChR currents in LVI interneurons were strongly desensitized (Student's t-test, $p<0.01$) in contrast to nAChR currents in LVI pyramidal neurons, which remain partially available for activation ($p<0.01$, indicated with $). nAChR currents in adolescent layer VI pyramidal neurons desensitized ($p<0.01$) to a similar degree as in juvenile mice ($p=0.64$). β2* nAChRs currents of LVI interneurons desensitized more than LV interneurons ($p<0.01$, indicated with $). In addition, β2* nAChR currents of LII-III interneurons desensitized stronger than layer VI pyramidal neurons ($p=0.02$, indicated with $). 100 nM nicotine also strongly desensitized β2* nAChRs of adolescent LVI pyramidal neurons ($p<0.01$, indicated with *), but less compared to layer II-III ($p=0.04$)

postsynaptic currents (EPSCs) (Figure 3C1 and C3, control n=21, 992 ± 172 %, $p<0.01$, nicotine n=11, 340 ± 34 %, $p<0.05$, nicotine vs control, $p<0.05$). This reduction was more prominent with a higher dose of nicotine (Figure 3C2 and C3, n=6, 118 ± 12 %, $p=0.96$, control versus nicotine, $p<0.05$). Taken together, these data show that in PFC LV, nicotine partially interferes with β2* nAChR signaling on NFS interneurons and glutamatergic inputs received by LV pyramidal neurons.

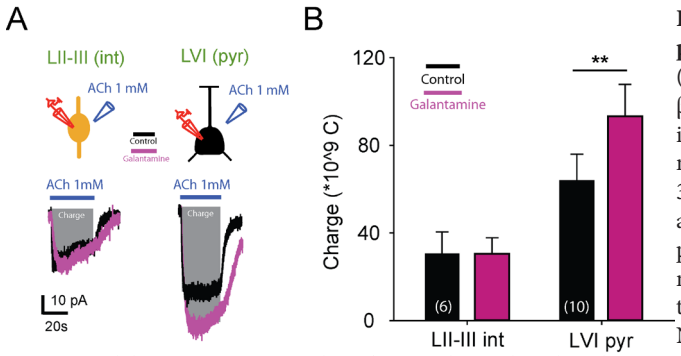


Figure 5. Galantamine does not potentiate LII-III β2* nAChRs.
(A) The effect of galantamine on β2* nAChRs was tested on LII-III interneurons and LVI pyramidal neurons. ACh was applied for 30 seconds before (black traces) and after galantamine (1 μM, pink traces) was washed in for 10 minutes. The lower panels show the average response for LII-III NFS interneurons (n=6) and layer VI pyramidal neurons (n=10). The effect on the ACh induced currents was assessed by calculating the total charge during the 30 second ACh application.
(B) Galantamine potentiates β2* nAChR currents on layer VI pyramidal neurons (Student's t-test, $p<0.01$), but not in LII-III interneurons ($p=0.97$).

Differential desensitization of β2-mediated nAChR currents in layer VI*

Layer VI pyramidal neurons are relatively spared from desensitization because of the presence of α5 subunits (Bailey et al., 2010). Whether this holds true for LVI interneurons, which are also modulated by β2* nAChRs (Figure 4A) (Poorthuis et al., 2013), is not known. To investigate possible differences we targeted these two cell types. A 10 minute application of nicotine completely abolished β2* nAChR-mediated responses to ACh application on non-fast spiking interneurons (Figure 4B, 4C and 4F, 13.1 % ± 3.4 remaining charge, n=7, $p< 0.01$). In contrast, β2* nAChR-mediated responses to ACh application of pyramidal neurons (Figure 4B) did not desensitize completely (Figure 4B, 4D and 4F, 29.1 % ± 3.1 remaining charge, n=5, $p< 0.01$). The degree of desensitization was significantly less for LVI pyramidal neurons compared to interneurons in LVI ($p<0.01$). During development, expression of nAChR subunits in LVI pyramidal neurons changes (Kassam et al., 2008). In the adolescent PFC, ACh-induced currents in LVI pyramidal neurons showed a similar degree of desensitization when exposed to nicotine as in the juvenile PFC (Figure 4E and F, 33.6 % ± 8.5 remaining charge, n=5, $p< 0.01$; juvenile vs. adolescence, $p=0.64$). LVI interneurons showed significantly stronger desensitization of ACh-induced β2* responses than LVI pyramidal neurons (Figure 4F, $p<0.01$) and LV interneurons (Figure 4F, $p<0.01$). Desensitization of β2*nAChR-mediated ACh-induced currents by nicotine was also significantly stronger in LII-III interneurons than in pyramidal neurons in LVI ($p=0.02$). Lower nicotine levels (100nM) also had a desensitizing effect on β2* responses of LVI pyramidal neurons (Figure 4F, 43.21 % ± 8.5 remaining charge, n=5, $p<0.01$), but less compared to layer II-III interneurons ($p=0.04$). These data show that layer-specific interference with cholinergic signaling also holds true for lower concentrations of nicotine.

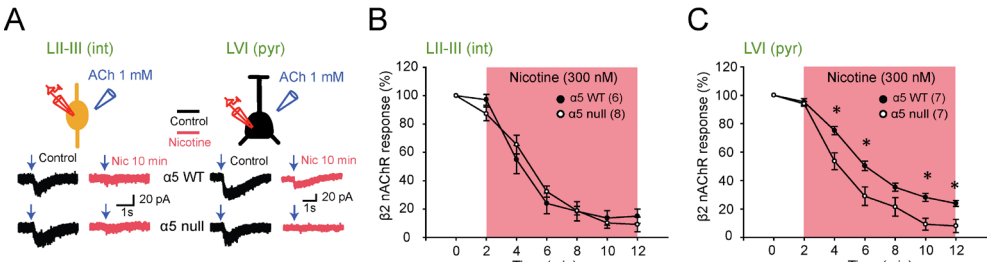


Figure 6. Expression of a5 nAChR subunits explains layer-specific desensitization of β2* nAChR currents by nicotine.

(A) Recording setup of experiment and example traces of nAChR currents in PFC LVI pyramidal neurons and in LII-III interneurons of wildtype and α5 null littermates before and after exposure to nicotine (10 min, 300 nM).

(B) Average response of β2* nAChRs on LII-III NFS interneurons to ACh stimulation (1 mM) in wildtype and α5 null adolescent mice. The degree of desensitization was not different for any timepoint in the absence of the α5 subunit (p>0.05 for all timepoints).

(C) Average response of β2* nAChRs on LVI pyramidal neurons to ACh stimulation (1 mM) in wildtype and α5 knockout adolescent mice. The degree of desensitization in the absence of the α5 subunit was faster (@ 2 min, Student's t-test, p=0.01) and stronger (@ 10 minutes, p=0.01)

Involvement of α5 nAChR subunit explains layer-specific interference of nicotine with cholinergic signaling

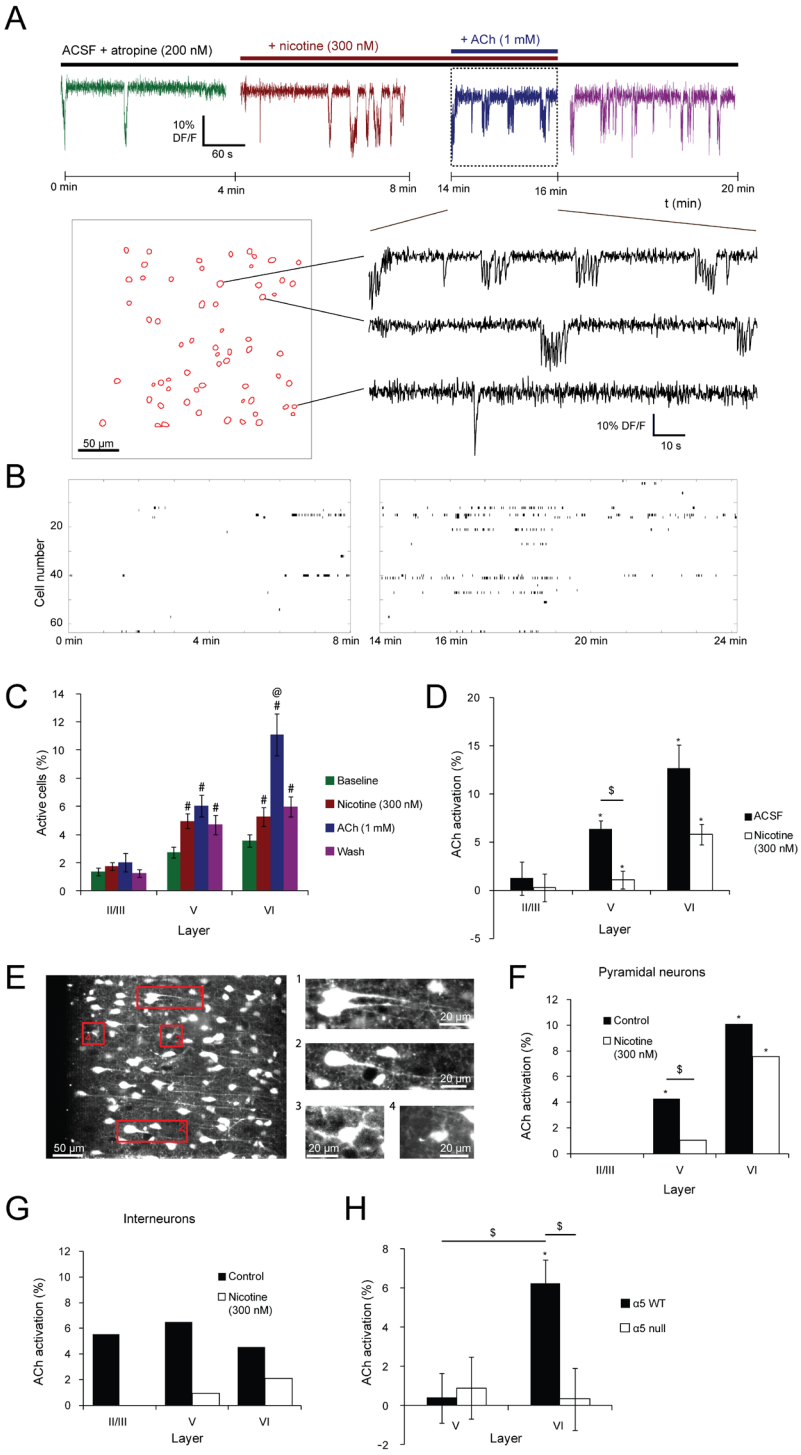
The level of desensitization of β2*nAChR-mediated ACh-induced currents differed in different PFC layers. Layer VI pyramidal neurons express the accessory α5 nAChR subunit, which protects β2* nAChRs from complete desensitization (Kassam et al., 2008; Grady et al., 2012). We hypothesized that β2* nAChRs expressed by neuron types that showed stronger desensitization did not contain the α5 nAChR subunit. To investigate this, we first used galantamine, an allosteric modulator that potentiates β2* nAChRs containing α5 subunits, but not β2* nAChRs lacking the α5 subunit (Kassam et al., 2008; Kuryatov et al., 2008). We applied acetylcholine (1 mM) with a puff electrode for 30 seconds and repeated this procedure after 10 minutes exposure to galantamine (1μM) to test for possible potentiation in adolescent animals (Figure 5A). ACh-induced β2*-mediated currents in layer II-III interneurons were not potentiated by galantamine exposure (Figure 5A and B, n=6, 30.4 ± 10.1 *10^-9 vs. 30.5 ± 7.2 *10^-9 C, p = 0.97). In contrast, β2* nAChR currents in layer VI pyramidal neurons were potentiated after application of galantamine (Figure 5A and B, n=10, 64 ± 12 *10^-9 vs. 93.2 ± 14.6 *10^-9 C, p = 0.01). These data suggest that a layer specific receptor composition of β2* nAChRs exists in the prefrontal cortex. β2* nAChRs in layer II-III do not contain α5 subunits, whereas β2* nAChRs on layer VI pyramidal neurons do contain α5 subunits.

We next tested the hypothesis that the nAChR α5 subunit determines the different layer-specific degree of desensitization. We targeted layer II-III interneurons and layer VI pyramidal neurons in the PFC of adolescent α5 null mice and their wild type littermates (P34-P43). LVI pyramidal neurons lacking the α5 subunit showed

a faster and stronger degree of desensitization of ACh-induced currents by nicotine than wild type LVI neurons (Figure 6A). After two minutes of exposure to nicotine, desensitization of ACh-responses was significantly stronger in the α5 knockout neurons compared to wild type neurons (Figure 6C, wildtype versus knockout; 75.2 ± 2.9 vs. 53.7 ± 5.9, p=0.01). After ten minutes β2* nAChRs of layer VI pyramidal neurons were completely desensitized, while pyramidal neurons in wildtype mice remained partially available for ACh activation (Figure 6C, 23.9 ± 2.1% vs. 8.1 ± 4.5%, p=0.01). In layer II-III however, the degree of desensitization was not affected by the absence of the α5 subunit at any time point (Figure 6B, wildtype versus knockout; 54.9 ± 10 % vs 65.4 ± 6.1%, p=0.40 after two minutes nicotine and 15.0 ± 4.9 % vs 11.6 ± 5.4 %, p=0.65 after ten minutes of nicotine). These data confirm that α5 subunits are not expressed by LII-III neurons and therefore show a stronger degree of desensitization of β2* nAChR currents by smoking concentrations of nicotine.

Nicotine limits nAChR-mediated neuronal activation to layer VI pyramidal neurons

Nicotine strongly affects cholinergic activation of β2* nAChRs in a layer specific manner. Therefore we asked the question to what extent neuronal activation by ACh in the different layers would be affected by the presence of smoking nicotine concentrations. To test this, we used 2-photon imaging of fura-2 loaded PFC slices and bath applied nicotine (300nM) for 10 minutes before bath applying ACh (Figure 7A). Bath application of ACh mainly affects action potential firing in neurons by activating β2* nAChRs (Poorthuis et al., 2013). Nicotine application increased neuronal activity in layer V and VI of the PFC (Layer V, Fisher's LSD posthoc test, p<0.01, Layer VI, p=0.04, Figure 7B-C). In LII-III, after application of nicotine, subsequent application of ACh did not increase neuronal activity and the number of activated cells per slice was similar as control conditions (Figure 7C-D, p=0.82). In layer V, neurons were activated (p<0.01) by low concentrations nicotine and subsequent application of ACh slightly increased this activity (p<0.05, Figure 7B-C). In layer VI, application of ACh in the presence of nicotine prominently increased neuronal activity (p<0.001, Figure 7B-C). To address the question whether the remaining activation of neurons in deep layers were pyramidal neurons or interneurons, we identified from the high resolution z-stacks the imaged neurons as pyramidal neurons or interneurons (Figure 7E). Nicotine application strongly reduced activation of interneurons in the PFC (p=0.039, Figure 7F-G). The effect of nicotine on pyramidal neurons was layer-specific. Layer VI pyramidal neurons were the only cell type that still showed an increase in activation upon ACh application in the presence of nicotine (p<0.001, Figure 7F). Pyramidal neurons in layer II-III and layer V and PFC interneurons showed no significant subsequent activation by ACh (Figure 7F-G, p>0.05). Thus, nicotine concentrations experienced by smokers results in the loss of ACh modulation of pyramidal and interneurons in LII-III and LV. In the presence of nicotine, only layer VI pyramidal neurons will respond to fast ACh signaling.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ACSF + atropine (200 nM)
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Figure 7. Nicotine limits nAChR-mediated neuronal activation to LVI pyramidal neurons.
(A) Example of an experiment using network calcium imaging. Contours of Fura2-AM loaded neurons were detected after which traces from these neurons were extracted. Shown are calcium events before, during and after the application of nicotine (300 nM) and ACh (1 mM).
(B) Rasterplot of the activity of all neurons in a slice during the experiment.
(C) Average percentage of active cells per slice per minute. Nicotine (300 nM) increased activity in layers V and VI (Fisher's LSD posthoc test; layer V: $p=0.0012$; layer VI: $p=0.038$; significant effects indicated with #) but not in layer II/III ($p=0.71$). Subsequent application of ACh (1 mM) only resulted in a significant increase of the percentage of active cells in layer VI ($p<0.0001$; layer II/III: $p=0.82$; layer V: $p=0.11$; significant effect indicated with @).
(D) Nicotine pre-application (300 nM) reduced the activation by subsequent ACh (1 mM) application (all layers: $p=0.002$). This effects was significant for layer V ($p=0.00004$) but not for layer II/III ($p=0.4$) or layer VI ($p=0.09$). Despite this, there remained a significant activation in layer V and VI (aCSF layer V: $p=0.000001$; aCSF layer VI: $p=0.001$; nicotine layer V: $p=0.032$; nicotine layer VI: $p=0.035$), whereas activation in layer II/III remained nonsignificant (aCSF layer II/III: $p=0.22$; nicotine layer II/III: $p=0.74$).
(E) Projection of z-stack showing the morphology of imaged neurons
(F) Nicotine (300 nM) desensitized the response to ACh (1 mM) in layer V pyramidal neurons (LV vs LVI: $p=0.0036$; without nicotine: $p=0.0019$; with nicotine pre-application: $p=0.08$), whereas layer VI pyramidal neurons remain responsive (LV vs LVI: $p=0.66$; without nicotine: $p<0.001$; with nicotine pre-application: $p<0.001$).
(G) Nicotine (300 nM) desensitized the responses of interneurons to ACh (1 mM) throughout all layers ($p=0.039$).
(H) The absence of desensitization of layer VI pyramidal neurons is dependent on the α5 nAChR subunit. Mice lacking this subunit have a desensitized response to ACh (1 mM) after nicotine pre-application in both layer V and layer VI (layer V: $p=0.40$; layer VI: $p=0.64$) whereas their WT littermate controls still show significant activation by ACh in layer VI (layer V: $p=0.55$; layer VI: $p=0.004$). The interaction between genotype and layer was significant ($p=0.027$) and the activation in layer VI of the littermate controls was significantly bigger than the activation in layer VI of the α5 null mice ($p=0.004$) and in layer V of the WT animals ($p=0.001$).

To test whether the remaining activation of layer VI neurons depended on the presence of the α5 subunit we imaged slices from α5 knockout and wildtype littermates. As shown in the previous experiment, there was a stronger activation of layer VI compared to layer V in wildtype mice (Figure 7H, $p<0.01$). In α5 null mice, ACh did not increase activity in layer VI in the presence of nicotine (Figure 7H, $p=0.64$) and ACh-induced activity was strongly reduced in PFC layer VI of α5 null mice compared to wildtype mice (Figure 7H, $p<0.01$). Hence, these data show that exposure to low concentrations of nicotine limits neuronal activation by cholinergic signaling through β2* nAChRs in the PFC to layer VI pyramidal neurons that express α5 subunits.

Discussion

In this study we showed that nicotine strongly reduces cholinergic activation of the PFC network and that this effect is cell type and layer specific and depends on nAChR subunit expression. Cholinergic responses mediated by β2* nAChRs desensitize after 10 minute exposure to smoking concentrations of nicotine (300 nM). In contrast, α7 nAChRs remained available for cholinergic signaling throughout the PFC circuitry.

β2* nAChR currents in interneurons in LII-III and LVI were completely desensitized by nicotine. β2* nAChR currents in LV interneurons were less compromised by nicotine exposure, just as β2* nAChR currents in LVI pyramidal neurons. Also β2* nAChRs on thalamic terminals activating layer V pyramidal neurons were strongly desensitized by nicotine. A similar degree of desensitization was found in adolescent animals, a developmental time period in which the PFC is vulnerable for long-term adaptations induced by nicotine (Counotte et al., 2011b; Goriounova and Mansvelder, 2012a). Layer-dependent desensitization of β2* nAChR currents in adolescent mice was caused by the presence or absence of α5 subunits. In conclusion, nicotine greatly reduced cholinergic activation and altered the balance of cholinergic signaling through nAChRs in the PFC neuronal network depending on nAChR subunit composition.

Cigarette smoking leads to a prolonged presence of nicotine levels in the brain that reach 300 to 600 nM for minutes (Matta et al., 2007). Smoking of one cigarette leads to nearly complete β2* nAChR receptor saturation in humans (Brody et al., 2006). Sustained exposure to low levels of nicotinic agonists rapidly desensitizes nicotinic receptors (Fenster et al., 1997; Picciotto et al., 2008). Whether smoking nicotine concentrations influence nAChRs by desensitization in circuits involved in attention behavior was not known. We find that nicotine rapidly decreases responsiveness of β2* nAChRs in the PFC, while leaving α7* nAChRs intact. Because of co-application of ACh and nicotine we cannot rule out agonist competition at the receptor binding site, however the persistent reduced responsiveness of β2* nAChRs (over 45 minutes) after the presence of nicotine suggests that nicotinic receptors indeed were desensitized. An alternative explanation could be that nicotinic receptors were internalized (John and Gordon, 2001). However, the responses did recover after an hour, suggesting recovery from desensitization. The subunit specificity of receptor desensitization observed is similar to that seen in the ventral tegmental area where nicotine desensitizes β2* nAChRs on GABAergic interneurons, but not α7 nAChRs on glutamatergic terminals and dopamine neurons (Mansvelder et al., 2002; Wooltorton et al., 2003). Hence, whereas α7 nAChRs display rapid desensitization kinetics after being activated by rapid increases in agonists, they do not desensitize upon the prolonged presence of smoking concentrations of nicotinic agonist. These separate processes, referred to as 'classical' and 'high-affinity' desensitization (Giniatullin et al., 2005), thus operate in the PFC as well suggesting that α7 nAChRs remain available for activation by fast cholinergic transients (Parikh et al., 2007).

The desensitizing properties of β2* nAChRs are heterogeneous. The accessory α5 subunit plays a critical role in determining whether β2* nAChRs remain available for cholinergic signaling (Bailey et al., 2010; Grady et al., 2012). In the cortex α5 subunits are preferentially expressed by neurons in deep layers (Winzer-Serhan and Leslie, 2005). Expression of α5 subunits is lower in superficial layers (Winzer-Serhan and Leslie, 2005), but still α5 could be located on NFS interneurons, which constitute a

small number of cells in the PFC modulated by β2* nAChRs (Poorthuis et al., 2013). In the PFC, α5 and β2 subunits co-assemble in LVI pyramidal neurons (Bailey et al., 2010). We find that the presence of α5 subunits does not extend to NFS interneurons in layer VI, which show a higher and complete degree of desensitization after nicotine exposure. However, it has been reported that some cortical interneurons express β2 and α4 subunits in combination with α5 subunits (Porter et al., 1999). We find that β2-mediated responses in LV interneurons show similar levels of desensitization as responses by LVI pyramidal neurons, suggesting that they may also express α5 subunits.

Exposure to nicotine during adolescence has perturbing effects on attention performance in later life (Counotte et al., 2011a). We investigated nicotine's effect on cholinergic signaling in the juvenile (P14-P21) and adolescent mouse (P34-P43). Although β2*, but not α7, nAChR receptor expression changes with age (Kassam et al., 2008; Counotte et al., 2012), we find similar percentage of β2* nAChR desensitization in both age groups. Receptor desensitization and strong interference with cholinergic signaling by concentrations of nicotine experienced by smokers may be the first step in a cascade of events leading to molecular, cellular and functional changes in the PFC. After adolescent nicotine exposure, the nicotinic receptor subunits α4 and β2 are strongly upregulated, whereas α7 and α5 subunit expression remains unchanged (Counotte et al., 2012). One may hypothesize that the strong desensitization of receptors containing the β2 subunit induces the upregulation following adolescent exposure as an adaptive strategy to maintain cholinergic signaling through these receptors. Similarly, the lack of desensitization of α7 nAChRs and the limited desensitization of α5 containing nAChRs do not trigger the upregulation. Indeed, after repeated nicotine exposure during adolescence, cholinergic control over GABAergic inhibition in LII-III is increased (Counotte et al., 2012), suggesting an augmentation of functional nicotinic receptors. Whether nAChR upregulation in the PFC after nicotine exposure during adolescence is cell-type and layer specific remains to be investigated. An increase in number of nAChRs at neuronal surfaces after prolonged nicotine exposure is probably mediated by several posttranslational mechanisms (Goriounova and Mansvelder, 2012a; Govind et al., 2012). Ultimately, compensatory mechanisms secondary to altered cholinergic signaling might lead to reduced mGluR levels and consequently alters synaptic learning rules and attention behavior (Counotte et al., 2011a, 2011b; Goriounova and Mansvelder, 2012b).

Although acute exposure to nicotine has been shown to enhance attention performance in rats under some circumstances (Hahn et al., 2003; Levin et al., 2006), nicotine has been found to decrease attention performance in mice (Bailey et al., 2010). Our integrative network approach shows that nicotine concentrations seen by smoking limits nAChR-induced action potential firing to layer VI pyramidal neurons.

What could be the functional consequence of this shift in cortical computation? Fast cholinergic transients are important for cue detection and attention behavior (Parikh et al., 2007, 2010). Nicotine exposure strongly abolishes control over GABAergic circuitry in the PFC. Nicotinic receptor activation of interneurons has been shown to modulate pyramidal neuron activity and increases the threshold for induction of spike-timing dependent synaptic plasticity in cortex and hippocampus (Ji et al., 2001; Couey et al., 2007). Cholinergic signaling might therefore increase the signal to noise ratio in the PFC. When nicotine is present in the PFC, this mechanism is absent and might lead to compromised information processing. At the behavioral level a lack of functional β2* nAChRs has been shown to lead to a hyperactive medial prefrontal cortex and altered social and exploratory behavior (Avale et al., 2011; Bourgeois et al., 2012), suggesting that the PFC network is disinhibited in the absence of this receptor. Supporting this, genetic deletion of β2* nAChRs also leads to impaired attention behavior, which depends on β2 subunits in the medial PFC (Guillem et al., 2011).

Nicotine-induced desensitization also reduced nAChR-mediated control over excitatory elements in layer V and VI. In the absence of nicotine, activity of pyramidal neurons in layer V is strongly enhanced by glutamate release induced by β2* nAChRs on axonal terminals originating in the medial dorsal thalamus (Lambe et al., 2003; Parikh et al., 2008; Poorthuis et al., 2013). The reduction in cholinergic nAChR-mediated control over this circuitry in the presence of nicotine might compromise cue-induced cholinergic transients and hence signal detection during attentional tasks (Parikh et al., 2010). Cholinergic induced activity of layer VI pyramidal neurons is also reduced. Part of the output neurons in layer VI form a thalamocortical loop (Kassam et al., 2008) and are important for regulating sensory presentations in the cortex (Olsen et al., 2012). Therefore, a decrease in cholinergic control of this circuitry might interfere with optimal attention performance (Bailey et al., 2010). In conclusion, nicotine leads to strong interference with cholinergic control over β2* nAChRs in the PFC which might compromise attention behavior on the short-term and leads to maladaptive changes of PFC circuitry which leads to altered attention behavior on the long-term.

General discussion

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Chapter 6

Published in Biochemical Pharmacology 2013 Jul 12
Nicotinic acetylcholine receptors controlling attention:
Behavior, circuits and sensitivity to disruption by nicotine
Poorthuis RB, Mansvelder HD

Introduction

Acetylcholine is a neuromodulator that acts in many brain regions to modulate a diverse repertoire of behavioral states (Picciotto et al., 2012). In the prefrontal cortex acetylcholine plays a central role in regulating attention (Sarter et al., 2009). Acetylcholine is released through axonal projections across the entire cortical mantle that originate in the basal forebrain (Woolf, 1991). In Alzheimer's patients these neurons decline, which leads to memory impairments and a loss in the ability to pay attention (Whitehouse et al., 1981; Perry and Hodges, 1999). Patients with Alzheimer's are treated with acetylcholine-esterase inhibitors, leading to a global increase in acetylcholine levels in the brain. These treatments are limited in their efficacy and lead to side effects like nausea and diarrhea (McGleenon et al., 1999). Also in animal models strongly increasing acetylcholine levels does not lead to improved attention (McGaughy and Sarter, 1998). It is therefore of critical importance to disentangle the specificity of the cholinergic system in orchestrating the range of cognitive functions it can affect. Ultimately this allows moving away from traditional pharmacological interventions that affect receptors in many different brain areas. Promising avenues to unravel specificity of the cholinergic system include (i) the description of brain circuitry recruiting cells in the basal forebrain involved in different tasks, (ii) defining causal relationships between the many receptor subtypes of the cholinergic system and a particular behavior and (iii) how these receptors alter computational properties of the neuronal network to support these behavioral functions. The rapid evolvement of genetic tools facilitated these important advances (Garner and Mayford, 2012). Ultimately, this will lead to more specific strategies to treat brain disease.

In the work described in this thesis we show for the first time a causal role for β2* nicotinic receptors in attention behavior and we specified the prelimbic cortex as its locus of action. Zooming into the network we show that this receptor plays a prominent role in regulating the inhibitory tone in the prefrontal cortex. In addition, it modifies information transfer between the cortex and thalamus. In this chapter I aim at linking the behavioral findings to nicotinic receptor modulation of prefrontal cortical cell types. By placing these findings in the context of the literature, I propose that these cell types play an important role in regulating integration of incoming information and orchestrate appropriate output of the prefrontal cortical circuitry. Furthermore I hypothesize how these top-down mechanisms might support attentional function through interactions with limbic circuits.

Nicotine in tobacco smoke acts on the cholinergic system through nicotinic receptors and thereby brings it out of balance. Smokers usually initiate their habit during adolescence. Nicotine can improve or decline attention performance on the short-term, while exposure during adolescence on the long term impairs attention performance. We show that nicotine has ambivalent effects on prefrontal neuronal networks. With progression of time nicotine activates and then desensitizes nicotinic

receptors, mainly affecting β2 receptors. In the last part of the discussion I argue how the initial boosting and subsequent diminishment of nicotinic receptor function alters prefrontal cortical function on the short-term and how the network adapts to the presence of nicotine on the long-term. I will place these findings in the context of how this affects attention behavior. A summary of the findings described in this thesis can be found in figure 1.

Contribution of different nAChR subunits to attention behavior

Cholinergic signaling in the prefrontal cortex plays a central role in regulating cue detection and attention behavior (Passetti et al., 2000; Parikh et al., 2007). Acetylcholine levels fluctuate on different time-scales to encode different behavioral relevance. Slow cortex-wide fluctuations in ACh levels predict whether a cue will be detected and lead to a shift in behavior towards reward retrieval. Fast cholinergic transients are seen during actual cue-detection and mediate the incorporation of a cue into new goal directed behavior (Parikh et al., 2007). Cholinergic signaling is necessary for proper attention performance. In the absence of cholinergic signaling neuronal activity related to performance in attention tasks is diminished, showing that acetylcholine plays a central role in encoding relevant activity in the prefrontal cortex to facilitate proper attentional performance (Gill et al., 2000). An unanswered question remained which receptors are involved in regulating attention and how they alter network activity within the PFC.

Nicotinic receptors are fast ionotropic receptors (McGehee and Role, 1995) and therefore a likely candidate to follow rapid changes in cholinergic signaling. In addition, nicotine alters attention behavior of rodents which is partly regulated through PFC dependent mechanisms (Hahn et al., 2003). Therefore we hypothesized that nicotinic receptors are critically involved in regulating attention. Lesions of the basal forebrain lead to strong impairment of attention behavior in the 5-choice serial reaction time task (5-CSRTT)(Muir et al., 1995; Risbrough et al., 2002). Therefore, we used this paradigm to assess the effect of genetic deletions of nicotinic receptors on attention behavior. In chapter 2 we show that deletion of the β2 subunit leads to strong impairment of attentional performance. β2 knockout mice show a higher level of omissions compared to their wildtype littermates, while their accuracy level is not affected. Hence, in the absence of β2 subunits mice respond less to the presented cue, but when they actually respond they do this in the correct nosepoke hole. We conclude that the absence of the β2 subunit leads to a gross impairment in attention, rather than having an effect on spatial choice discrimination.

Do the higher omission levels in β2 knockout mice actually reflect a deficit in attention? Since knockout mice lack receptors throughout their brain several behavioral factors could be compromised (Gotti and Clementi, 2004; Changeux, 2010) that can lead to altered outcomes in the 5CSRTT (Robbins, 2002). Nicotinic

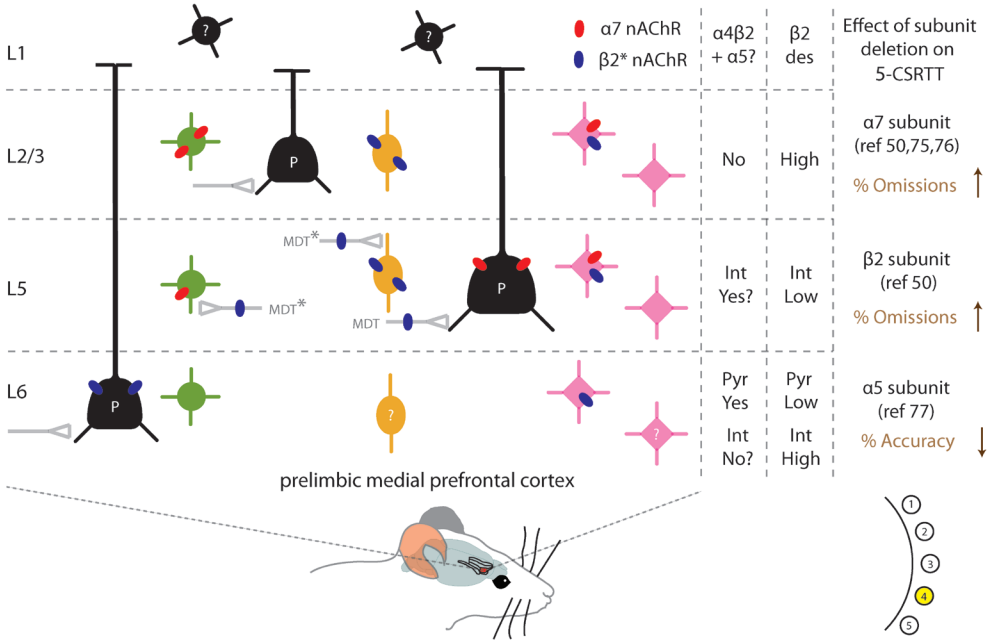

Figure 1. Overview nicotinic receptor modulation of prefrontal cortical circuitry and the 5-CSRTT. Left. Schematic representation of the α7 and β2* nAChR distribution across neurons in the layers of the prelimbic PFC. In superficial layers, only interneurons are modulated by nicotinic receptors, while in deeper layers pyramidal and interneurons contain nAChRs (Based on [66, 68, 79]). Black cells = pyramidal neurons, green cells = fast-spiking interneurons, orange cells = somatostatin positive interneurons, pink cells = regular-spiking non–pyramidal neurons, grey lines = glutamatergic synapses, MDT = medial dorsal thalamus. ? Indicates the presence of nAChRs has not been tested. * Presumably glutamatergic inputs from the MDT. Right. α4β2 + α5?; In this table it is indicated in which layers and on which cell types β2* nAChRs contain the accessory α5 subunit (pyr = pyramidal neurons, Int = interneuron) [77, 80]. [50, 75-77]. ? Indicates that the presence or absence of α5 subunits is not directly tested with galantamine, but is predicted to be present or absent based on the differential degree of desensitization when exposed to nicotine as observed in Poorthuis et al. 2013. β2 des; Indicated is the degree of desensitization of β2* nAChRs in the different layers and cell types in the prelimbic cortex when exposed to smoking concentrations of nicotine (300 nM, High/Low indicates the relative degree of desensitization among β2* nAChR populations [77, 80]). Most right table displays the effects on 5-choice serial reaction time task (5-CSRTT) performance after genetic deletion of nAChR subunits. Up and down arrows indicate an increase or decrease in the attentional parameter indicated.

β2 receptors play a major role in motivational behavior (Maskos et al., 2005) and in associative learning (Conner et al., 2003; Letzkus et al., 2011). However, β2 knockout mice show no impairments in spatial memory acquisition (Cordero-Erausquin et al., 2000). In addition, in the 5-CSRTT β2 knockout mice show no effects on omission levels at longer stimulus duration, indicating that they learned to associate the cue with the reward. In spite of normal acquisiton, there was no sign of recovery from

attention impairment over sessions at shorter stimulus duration. This indicates that the absence of the β2 receptor leads to permanent impairments in attention rather than learning. These results are in line with studies showing consistent decrements in attention after cholinergic lesions in the basal forebrain even after extensive training (McGaughy et al., 1996). Arguing against this, blocking nicotinic receptors in mice after acquisition did not affect errors of omission or any other measure of attention (Pattij et al., 2007). However, this might be due to a ceiling effect caused by high baseline omission rates. Difference in omission levels are strongly interrelated with motivation of animals to perform the task (Robbins, 2002). β2* nicotinic receptors are highly expressed in dopaminergic neurons of the ventral tegmental area where they regulate rewarding properties of the drug nicotine (Maskos et al., 2005). Hence the increase in omission might indicate a lack of interest to earn rewards. Several findings however argue against this view. First the latency to collect rewards was not different between genotypes. In addition, we showed that both genotypes were also equally eager to earn rewards when they could earn rewards unlimitedly by making a single nosepoke or in a progressive ratio schedule for reinforcement. This shows that under low attentional demands basic motivation to earn rewards is not altered. Lastly, also locomotor activity was not affected, since reaction time for correct responses was not different between genotypes and no difference was found in an open field test for locomotor activity. Hence we conclude that in the 5-choice task genetic deletion of β2 leads to impairment of attention behavior. This conclusion is in line with studies lesioning the cholinergic system, which also lead to attention impairments without affecting locomotor or motivation behavior (Risbrough et al., 2002; Dalley et al., 2004). Substantiating this, cortical lesions of cholinergic inputs increase omission rates (Risbrough et al., 2002) and the level of omission is inversely correlated to the amount of ACh efflux into the PFC (Passetti et al., 2000). However other studies show impairments in accuracy after ACh lesions making it hard to interpret the precise role of acetylcholine in orchestrating attention. Using optogenetic strategies to deplete the PFC from cholinergic signaling in β2 knockout mice can reveal muscarinic receptor effects on attention performance.

A question remaining regarding the effect of acetylcholine on attention is which brain circuitries mediate the effects of acetylcholine on attention. If cognitive enhancing strategies are to be designed to ameliorate for example attention deficits of Alzheimer patients, a more precise localization of receptor subtypes involved in attention is inevitable. In particular because increasing overall acetylcholine levels does not prove to be an effective strategy in increasing attention in humans (Benzi and Moretti, 1998; McGleenon et al., 1999) and rats (McGaughy and Sarter, 1998). Genetic strategies using viral vectors to deliver a single gene in a target region proved useful in investigating these questions (Maskos et al., 2005; Fowler et al., 2011). Although cortical fluctuations of acetylcholine manifest throughout the cortex , during cue detection acetylcholine is released specifically in the medial PFC (Parikh

et al., 2007; Sarter et al., 2009). Together with lesion studies showing decreases in attention upon prefrontal cortical cholinergic depletion (Dalley et al., 2004), it makes the medial prefrontal a likely candidate for mediation of cholinergic signaling in attention behavior. However, a causal role for cholinergic signaling in the mPFC, in particular in relation to a specific receptor, was never proven.

To answer this question we reexpressed β2 subunits in the prelimbic area of the prefrontal cortex (Chapter 2) (Guillem et al., 2011). Somewhat surprisingly, reconstitution of the receptor only in postsynaptic neurons of the prefrontal cortical circuitry fully restored the deficits in attention (for an overview of nicotinic receptors in PFC see figure 1). Presynaptic nicotinic receptors are found on thalamic afferents to the PFC (Gioanni et al., 1999; Lambe et al., 2003) and specifically activate layer V neurons (Chapter 3). Stimulation of these β2 nAChRs leads to enhanced cholinergic release (Parikh et al., 2010), which correlates to behavioral shift offset after cue detection (Parikh et al., 2007). Stimulating β2 receptors enhances cue detection (Howe et al., 2010) and it has been hypothesized that this is regulated by glutamatergic-cholinergic interaction through thalamic terminals (Howe et al., 2010). Our experiments indicate however that not presynaptic nAChRs but postsynaptic nAChRs in the PFC play a major role in performance on the 5-CSRTT. This conclusion is drawn with caution, because nicotinic receptor knockout mice show prominent upregulation of muscarinic receptors which rescues neuronal activation partially (Tian et al., 2012). Hence, this might have masked an additional β2* receptor contribution by elevating the muscarinic component contributing to attentional performance. Conditional knockout strategies are a useful tool to define more sensitively the effects of nicotinic receptor deletion on attention in the absence of developmental effects (Sauer, 1998). In general, these findings do not prove sufficiency of β2* receptors in mediating cholinergic signaling involved in attention. Other nicotinic receptor subunits might also be involved in attention (see below, (Young et al., 2004; Young et al., 2007; Bailey et al., 2010). In addition, muscarinic receptors play an important role in attention behavior and cue detection (Pattij et al., 2007; Gulledge et al., 2009). However it suggests that the main focus of action of β2* nicotinic receptors on attention lies within this medial prefrontal circuitry. Hence despite the fact that part of cholinergic signaling manifests cortex wide (Parikh et al., 2007) and task associated activity is seen in all mPFC areas (Totah et al., 2009), β2* receptor functioning in attention is centered in the prelimbic cortex.

This poses the question whether other nicotinic receptor subunits contribute to attention and how β2 subunits regulate the prefrontal cortical circuitry to enhance attention performance on the 5-CSRTT. Next to β2 subunits, nAChR subunits α7 and α5 are particularly highly expressed in the mPFC (Counotte et al., 2012b). Under the same task conditions we did not find an effect of α7 genetic deletion on performance in the 5-CSRTT. This is in contrast to earlier reports showing delayed task acquisition and reduced omission levels at baseline performance (Young et al., 2004). The effect

on acquisition was however not seen in a follow up study by the same group and hence might have been caused by heterogeneity of genetic constitution due to limited backcrossing (Young et al., 2007). Supporting the fact that α7 receptors play a role in attention, this study showed again an increase in omission levels under high attentional load in α7 knockout mice (Young et al., 2007). Increasing attentional load in our study by randomly decreasing stimulus duration did not reveal any attentional deficits. The discrepancy might arise from the increased session duration in the study by Young et al., which results in more attended trials and therefore might have taxed sustained attention more than in our paradigm. In addition, the use of a liquid sucrose reward also seems to lead to an increase in attended trials causing bigger demands on sustained attention across sessions (Young et al., 2007; Bailey et al., 2010). Together, we conclude that both α7 and β2* receptors contribute to attention behavior. The parallel experiments on α7 and β2 knockout mice allow the conclusion that the involvement of β2* receptors is more robust, since deficits in attention are revealed under conditions with lower demands on attention which show no deficit in α7 knockout mice.

Interestingly the α5 subunit of the nicotinic receptor has also been reported to affect attention performance (Bailey et al., 2010). The α5 subunit is an accessory subunit that coassambles together with the β2 and α4 subunit to form receptor complexes (Salas et al., 2003). The α5 subunit gives the receptor a higher affinity for acetylcholine. Mutations of the α5 subunit are associated with a higher incidence of smoking and it has been suggested that this is partly due to cognitive deficits. Therefore it is of particular interest to study the role of this subunit and its mutations in attention (Wang et al., 2009). Deletion of the subunit leads to a less prominent activation of layer VI pyramidal neurons in the mouse mPFC (Bailey et al., 2010). Surprisingly, in the absence of the α5 subunit mice are less accurate while performing the 5-CSRTT under high attentional demand. This provokes the question how different nicotinic receptor subunits, constituting similar type of receptors, can affect different attentional modalities. Several hypotheses can be put forward to tackle this question. First, since α5 subunit deletion only diminishes signaling through β2 nAChRs, cells expressing this type of receptor might still follow cholinergic signaling involved in cue detection, but this might not be sufficient to properly detect and locate the cue. As a result cholinergic signaling is sufficient to detect and process the cue and does not lead to an error of omission, since detection alone can initiate a behavioral response. However under high taxing conditions cholinergic signaling might not be sufficient to maintain response accuracy, leading to an decrease in accuracy levels. A second explanation might be that α5 subunits show a different distribution compared to β2 receptors in general, i.e. not every β2 receptor comprises a α5 subunit. In that case a different population of cells that might specifically regulate accuracy is affected by α5 subunit deletion. Alternatively, it might be that α5 subunits affect attention behavior in a different brain region. We set out in testing the involvement of the α5 subunit in the prelimbic area. In our paradigm α5 knockout mice did not show any

attentional effects under baseline conditions (SD1), whereas simultaneous assessment of β2 knockout mice did show an effect on omission as in previous experiments. An absence of effect on baseline performance was similar to what has been reported. However, also increasing the attentional load by increasing the number of trials and making the stimulus duration unpredictable did not lead to an accuracy deficit (Figure 2), in contrast to earlier reports. We conclude that the α5 subunit does not play a robust role in this attention paradigm as compared to knockout of the complete β2 receptor, which were assessed in parallel and again showed an effect on omission levels as described before. α5 Knockout mice show increased muscarinic activation of prefrontal cortical circuitry (Tian et al., 2012). Conditional knockout experiments can better define the role of the α5 subunit in attention. Optogenetic strategies expressing viruses under α5 and β2 promotors might reveal how different cell types expressing these subunits differentially modulate attentional domains.

β2 receptors and the orchestration of attentional circuits

Our data support the conclusion that β2* nAChRs have a dominant role in regulating attention behavior. A question that remains is how this receptor regulates cognitive functioning to support proper attention behavior. We hypothesize that the β2* nAChR within mPFC circuits is necessary for regulating topdown attention. Instead of regulating cue detection per se this receptor might be necessary to encode reward anticipation and might therefore be important for motivation to pursue goal-directed behavior which might be compromised under high demands on attention. β2* receptors translate cholinergic signaling into neuronal activation in the mPFC (Chapter 3). Cholinergic signaling fluctuates on different time scales to regulate cue-detection (Parikh et al., 2007). Two modes of cholinergic signaling are present. First, cortex wide cholinergic fluctutations can predict whether a cue is detected. Second, a sharp rise in acetylcholine levels is seen during a behavioral shift following cue-detection. Hence, these two mechanisms might act in concert to regulate topdown attention (Sarter et al., 2009). At present it is unknown how tonic and phasic cholinergic signaling contribute to performance in tasks with high demands on attention (Parikh and Sarter, 2008). Several observations allow and fundament an extrapolation of these findings to the 5CSRTT. First, during performance of the 5CSRTT neuronal firing alters when an animal is anticipating for a cue and this preparatory activity predicts behavioral outcome (Totah et al., 2009). Second, reducing cholinergic innervations of the mPFC ameliorates task associated activity in the PFC before and during cue detection (Gill et al., 2000). Cholinergic action might therefore be two-fold. First β2 receptors might respond to tonic fluctuations in acetylcholine supporting preparatory activity and making the cortex 'ready' for processing sensory input. Second, β2 receptors might respond to rapid rises in acetylcholine during cue detection.

To form a hypothesis on how β2 receptors exert their action to favor proper

attention behavior it is necessary to discuss what cholinergic transients 'encode' during cue detection. It is hypothesized that they facilitate decision making processes rather than encoding sensory input into the mPFC per se (Parikh and Sarter, 2008). This is substantiated by the finding that the latency between cue presentation and reward delivery determines the timing of the acetylcholine peak and gathering reward in the absence of cue-detection does not lead to transients itself. In addition, rats show short interruptions of grooming behavior when cues are missed. This orienting might indicate that cues enter sensory streams but do not lead to a shift in behavior (Parikh et al., 2007; Gulledge et al., 2009). Cholinergic transients therefore probably facilitate decision making and cause a shift in behavior towards a new future goal (Parikh and Sarter, 2008; Sarter et al., 2009). Therefore we hypothesize that β2 knockout mice show specific deficits in preparatory activity or in decision making upon cue-detection due to a difference in reward anticipation. Motivational differences might only appear on high attentional demand when behavior needs to be structured in time during individual trials and sustained across sessions. Therefore they could be absent when assessed during basic motivational tasks. We show in chapter 2 that postsynaptic nicotinic receptors within the mPFC circuit mediate attention behavior. β2 Receptors are also found on glutamatergic terminals of thalamic inputs in layer V (Gioanni et al., 1999; Lambe et al., 2003; Couey et al., 2007; Parikh et al., 2008). Since expression of β2 receptors in mPFC circuitry is sufficient to rescue cue detection, our data suggests that these receptors do not influence attention behavior in this paradigm. This conclusion is drawn with caution since upregulation of muscarinic receptors might mask an additive effect of this circuitry on attention (Tian et al., 2012), as discussed above. But available evidence at least suggest that this population of receptors is a major component in regulating attention and might in particular respond to fluctuations of acetylcholine before and during cue detection. Experiments investigating single-unit activity in wildtype and β2 knockout mice during 5-choice performance might reveal specific loss of task related activity following cue-detection. This experiment can reveal whether differences are seen in preparatory activity, encoding the willingness for sensory input processing, or during a shift in behavior (decision making) after cue-detection necessary to obtain reward. In addition, it would be interesting to investigate whether β2 knockout mice show similar scanning strategies to monitor reward ports in relation to preparatory activity and/or whether they display attentional orienting during cue-presentation but, despite that, fail to respond with a proper behavioral outcome.

Following this line of reasoning, it is interesting to know what neuronal systems are dysregulated after β2 deletion. Defining the precise construct and circuitry mediating attention is a dazzling task. Many brain areas are involved including prefrontal, striatal, thalamic and emotional circuits which dynamically interact to support attention (Robbins, 2002). Perturbation of these areas leads to diverse impairments of several parameters of the task, which are in some cases distinguishable but also many times

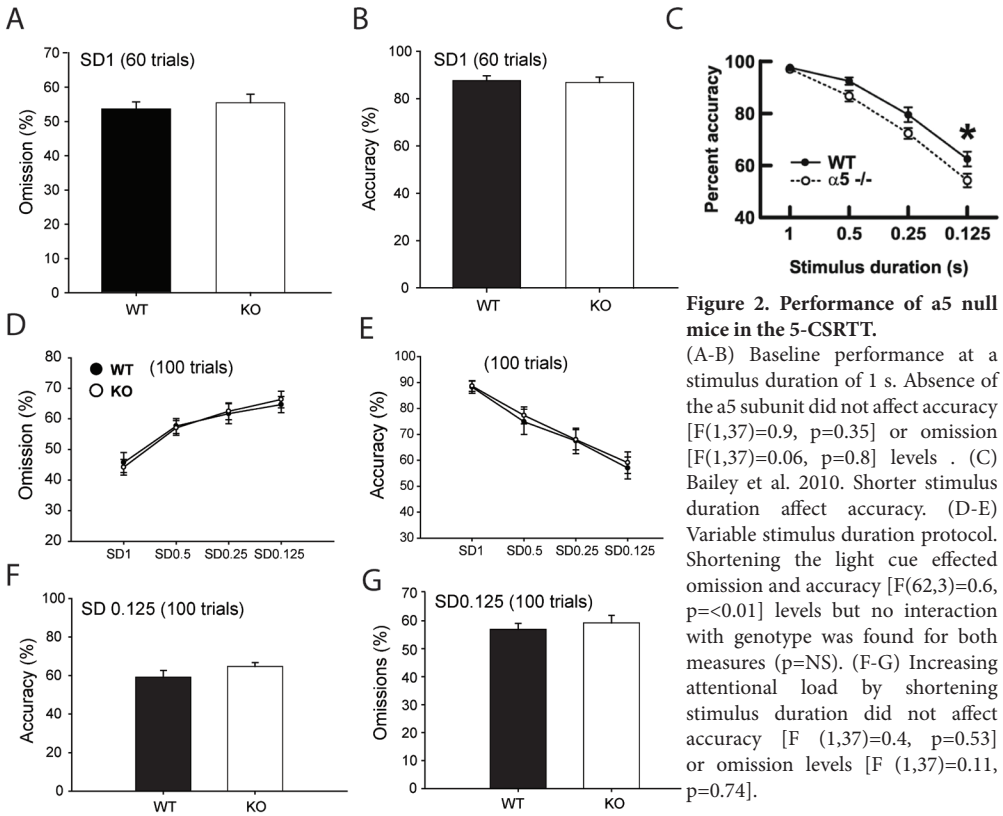

Figure 2. Performance of a5 null mice in the 5-CSRTT.
(A-B) Baseline performance at a stimulus duration of 1 s. Absence of the a5 subunit did not affect accuracy [F(1,37)=0.9, p=0.35] or omission [F(1,37)=0.06, p=0.8] levels . (C) Bailey et al. 2010. Shorter stimulus duration affect accuracy. (D-E) Variable stimulus duration protocol. Shortening the light cue effected omission and accuracy [F(62,3)=0.6, p=<0.01] levels but no interaction with genotype was found for both measures (p=NS). (F-G) Increasing attentional load by shortening stimulus duration did not affect accuracy [F (1,37)=0.4, p=0.53] or omission levels [F (1,37)=0.11, p=0.74].

overlap (Robbins, 2002). Evaluating the anatomical connectivity of the prefrontal cortex and functional overlap of impairments of lesion studies in the 5CSRTT can provide a framework for hypothesis and future experiments in an attempt to explain the role of β2* receptors in attention. The prefrontal cortex is a layered structure and projection neurons display preference for target structure dependent on the layer (Gabbott et al., 2005). Deep layers (mainly LVI) project to the medial dorsal thalamus (Gabbott et al., 2005) show specific expression of β2 receptors (Kassam et al., 2008) and lesions in the medial dorsal thalamus affect attention performance mainly through premature responding (Chudasama et al., 2001). Middle layers (mainly layer V) project to the ventral striatum (Gabbott et al., 2005), a structure highly involved in attention (Rogers et al., 2001). Superficial layers (mainly LII-III) project to the amygdala (Gabbott et al., 2005) and amygdala lesions show also impairments in attention performance (Holland et al., 2000). Hence, cortical-subcortical loops exist to facilitate attention performance (Groenewegen and Uylings, 2000). Our cortical mapping experiments in chapter 3 show that postsynaptic β2 can influence prefrontal cortical circuitry trough two main mechanisms; (i) by activating β2 receptors on pyramidal neurons projecting to the medial dorsal thalamus and (ii) through regulating inhibitory tone across all layers.

Lesions of the medial dorsal thalamus mainly affect premature responding, which are not seen after prelimbic lesions and hence suggests that a different thalamocortical circuit, perhaps to the cingulated cortex, might be important in inhibitory control (Chudasama and Muir, 2001). In addition thalamic lesions, but not prelimbic lesions, lead to enhanced error making during acquisition of a visual discrimination task (Chudasama et al., 2001). One can hypothesize that β2 receptors therefore mainly act through inhibition in the prefrontal cortex. Conditional expression of β2 receptors in these two separate populations of cells using the Cre-lox system in mice, expressing Cre under the CamKII promoter or the GAD2 promotor, is a useful tool in testing this hypothesis.

β2* Receptors are in a strategic position to regulate output of all cortical layers by increasing inhibition (Poorthuis et al., 2013b). Lesions of the striatum severly impair performance on the 5CSRTT (Rogers et al., 2001). Of the prefrontal cortical structures the prelimbic cortex has most dense projections to the ventral striatum, including the nucleus accumbens (Vertes, 2004), whereas cingulated cortex mainly projects to the dorsal striatum. Rescue of attentional deficits is seen after reexpression of β2 receptors in the prelimbic area, but not in the anterior cingulated cortex (Guillem et al., 2011). Interestingly dopamine depletion in the ventral and dorsal striatum mainly affects the willingness to respond. An increase in omission and response latency are the main factors compromised after 6-OHDA lesions on this task, while discriminative choice accuracy is mainly spared during these manipulations (Cole and Robbins, 1989; Baunez and Robbins, 1999). An effect similar to β2 knockout mice which also show an gross impairment in attention, having less willingness to respond (higher omissions) but show no impairments of discriminative choice accuracy or impulsivity (Guillem et al., 2011). I hypothesize therefore that activity in the cortico-striatal loop might be altered in β2 knockout mice that compromises attention performance. This might be due to a deficit in reward anticipation and consequently a failure to suppress irrelevant internally driven behavior, like grooming or exploration (Parikh et al., 2007; Bourgeois et al., 2012). Interestingly it has been shown that nucleus accumbens activity can in turn regulate acetylcholine release in the prefrontal cortex. Activating mesolimbic output of cholinergic projections can enhance cue detection and increases resistance to external distracters (St Peters et al., 2011). Hence, topdown glutamatergic inputs from the prefrontal cortex might be important in regulating motivational state to increase attention and goal-directed behavior over longer periods during challenging conditions (Gruber et al., 2009). The absence of cholinergic control over inhibitory circuits in the β2 knockout (Poorthuis et al., 2013b) might lead to altered output in the PFC-striatal network (see also below) because of hyperactivity in the circuit. A decrease in motivational state might lead to behavioral switching to internally driven behaviors like grooming or exploration. It would be of interest therefore to assess the relation between prefrontal cortical output and the ventral striatum. Optogenetic inhibition of PFC outputs to the ventral striatum using halorhodopsin / Archaerhodopsin or

measuring from optogenetically identified pyramidal neurons (Lima et al., 2009) in the PFC projecting to the ventral striatum are interesting strategies to resolve these questions. This will lead to a more refined view on regulating of attention processes by specific brain circuitries going beyond lesion studies.

Modulation of prefrontal cortical networks by nicotinic acetylcholine receptors

We showed in chapter 2 that β2 nAChRs in the prelimbic area play a prominent role in controlling attention (Guillem et al., 2011). This raises the question how this receptor alters cortical information processing in the underlying neuronal network to support attention performance. Unit activity in the mPFC alters during demands on attention. Cholinergic deafferentiation of the cortex strongly attenuates this task associated activity during attention performance (Gill et al., 2000). Interestingly, next to increased unit firing, a prominent part of cellular activity shows a decrease in activity (Gill et al., 2000), also during 5-choice performance (Totah et al., 2009). This indicates that only part of the network needs to be active to support attention performance and is in line with the hypothesis that the cortex functions through a tight excitation/inhibition balance. Mice lacking β2 subunits show a hyperactive mPFC during a cognitive exploration task (Bourgeois et al., 2012). This suggests that this receptor plays an important role in regulating appropriate firing levels in the mPFC. This mechanism might be important to surpress irrelevant task-distracting information. Indeed it has been shown that a diminishment in cholinergic signaling strongly impairs filtering out cross modal distracters (Newman and McGaughy, 2008). In addition, in appetitive cue-detection tasks a missed cue leads to an absence of cholinergic signaling. In this case animals orient towards the cue, but might fail to inhibit internally driven behaviors like grooming to respond to the cue. This might arise from a failure to suppress associational activity and be ready for input processing (Parikh et al., 2007; Parikh and Sarter, 2008). Suppression of irrelevant information and selection of one output neuron over the other might well be regulated by local inhibitory neurons within the network. In chapter three we describe a prominent role for β2* receptors in regulating inhibition in all prefrontal cortical layers. In this section we discuss computational roles of different type of interneurons and their regulation by nAChRs. We propose that β2* nAChRs play a key-role in regulating inhibition levels in the PFC which might support the suppression of irrelevant associational activity and selecting appropriate output of the mPFC circuit.

Role of inhibition in the cortex and its modulation by nAChRs

Balancing excitation with inhibition is critical for cortical function (Isaacson and Scanziani, 2012). An altered excitiation/inhibition ratio is thought to underlie psychiatric disorders like schizophrenia and autism (Kehrer et al., 2008; Gogolla et al.,

2009). Indeed, alterations in this ratio in the mPFC cause social dysfunction (Yizhar et al., 2011). However, temporal changes in excitation to inhibition ratio might support learning and allows direction of flow of information for example from layer II-III to layer V (Isaacson and Scanziani, 2012). The cortex is built up of functional microcircuits maintaining this balance and is determined by several configurations that exist to prevent overexcitation of the cortex. Afferent connections to cortical areas are arranged in a feedforward inhibitory circuit. In this configuration glutamatergic inputs from distant areas and from other layers synapse onto both pyramidal neuron and interneurons (Porter et al., 2001; Adesnik et al., 2012). This configuration is found in all cortical areas and mainly involves parvalbumin fast-spiking interneurons (Porter et al., 2001; Pouille and Scanziani, 2001; Rotaru et al., 2005). The same axon makes stronger glutamatergic connection to interneurons compared to pyramidal neurons ensuring that excitation is followed immediately by inhibition (Cruikshank et al., 2007). Excitation generated by principal neurons locally within cortical circuits is balanced by inhibition through feedback mechanisms (Isaacson and Scanziani, 2012). In the PFC, light activation of pyramidal neurons expressing channelrhodopsin-2 leads to strong inhibitory currents in neighboring pyramidal neurons (Figure 3). Connectivity between pyramidal and interneurons is reciprocal and abundant. Pyramidal neurons are mainly connected to each other through interneurons and can inhibit >50% of neighbouring pyramidal neurons (Silberberg and Markram, 2007; Fino and Yuste, 2011). The main interneuron subtype in generating disynaptic inhibition between pyramidal neurons is the Martinotti interneuron and is present in all cortical layers and areas (Kapfer et al., 2007; Silberberg and Markram, 2007; Berger et al., 2009). This pathway leads to horizontal inhibition within layers and is crucial in regulating cortical processing (Adesnik et al., 2012). A clear role has been established for parvalbumin and somatostatin expressing interneurons. Another class of regular-spiking non-pyramidal neurons, defined by CCK expression, is less well studied. Since it is not strongly recruited by feedforward or feedback circuitry we hypothesize that they are mainly recruited by neuromodulators, like nicotinic receptors (discussed below). In our studies (chapter 3 and 4) we found that interneurons are highly regulated by nicotinic receptors. Therefore nicotinic receptors are in a strategic position to alter information processing in the prefrontal cortex.

We used electrophysiological criteria, histochemical markers and genetic approaches to identify three main subtypes of interneurons in the mPFC. that the first group of interneurons fired non-adaptively at high frequencies, had short action-potential halfwidth and low input resistance and were classified as FS-interneurons. These interneurons never expressed β2* receptors. However, in contrast to earlier reports (Xiang et al., 1998; Couey et al., 2007; Gulledge et al., 2007) they expressed α7 receptors mainly in LII-III. Fast-spiking cells are known to target perisomatic compartments of pyramidal neurons and play a role in feedforward inhibition and integration of afferent inputs. Therefore, α7 receptors might have a modulatory role in

regulating feedforward inhibition of hippocampal or thalamic inputs (Tierney et al., 2004; Rotaru et al., 2005).

Interneurons are important in regulating dendritic excitability. Pyramidal cells are characterized by an apical dendrite extending into layer 1 to integrate incoming information across layers (Spruston, 2008). Dendritic compartments in the PFC are under differential control of interneurons, potentially altering integration of input at different layers (Kawaguchi and Kubota, 1997). Martinotti cells target distal dendrites in layer 1 (Silberberg and Markram, 2007) a rich source of incoming feedback information in the cortex (Larkum, 2013). Although distally located, they serve an important role in regulating pyramidal neuron output by regulating dendritic excitability. Inputs arriving in distal dendrites elicit dendritic action potential which is very powerful in driving the pyramidal neurons to spike (Larkum et al., 2001; Larkum and Zhu, 2002) and can be prevented by feedforward inhibition (Murayama et al., 2009). This mechanism serves an important role in encoding sensory stimuli in vivo (Murayama et al., 2009). Therefore Martinotti cells are in a perfect position to influence information integration in the PFC. Martinotti cells express the neuropeptide somatostatin, sometimes fire rebound spike after hyperpolarization and show adaptive spike frequencies (Goldberg et al., 2004). We identified somatostatin expressing cells based on their low-threshold spiking property and histochemical marker (LV) or using a genetic reporter line (LII-III)(chapter 3 and 4). In both layers somatostatin cells are regulated by β2* nAChRs and hence in part might account for the strong inhibition of the pyramidal network we observed during nicotinic receptor stimulation (Couey et al., 2007; Poorthuis et al., 2013b). Hence, β2* receptors play a central role in regulating feedforward inhibition which might serve to fine-tune processing of inputs arriving and selecting relevant output in the PFC network and potentially suppress irrelevant distracters. Hypothetically this could underlie maintenance of goal-directed behavior during attentional tasks which is compromised in β2 knockout mice.

A third group of interneurons we defined were the non-fast spiking (NFS) interneurons (Kawaguchi and Kubota, 1997). This group forms a heterogeneous group of cells including persomatic targeting basket cells expressing CCK and the aforementioned somatostatin expressing cells (Markram et al., 2004). The largest part of this group consists of regular-spiking non-pyramidal neurons (RSNP) whose firing profile resembles pyramidal neurons and shows slight adapting frequency and usually have a multipolar morphological appearance (Couey et al., 2007; Poorthuis et al., 2013b). Regular-spiking non-pyramidal neurons were found to express CCK (Couey et al., 2007). We found that this interneuron type expressed β2* receptors half of the time, and is sometimes accompanied by an α7 receptor. β2* Receptor expression of this cell type was found across all cortical layers, indicating that they perform similar roles across these microcircuits to fine-tune pyramidal function (Poorthuis et al., 2013b). While parvalbumin and somatostatin interneurons expressing interneurons have a well explored and described function, the role of RSNP or CCK positive

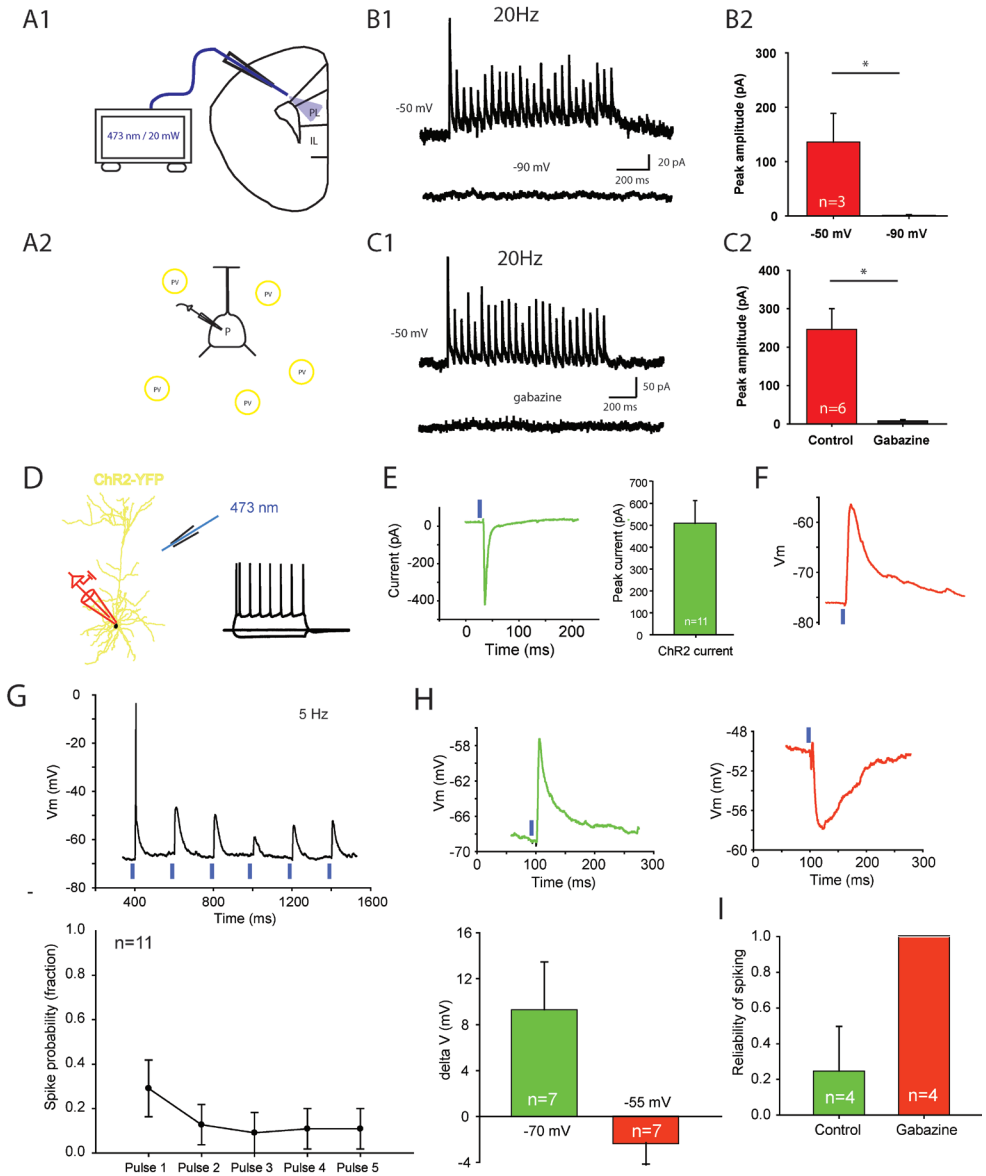

Figure 3. Optogenetic control over genetically identified cell types.

(A) mPFC neurons were transfected with a conditional ChR2 virus in mice that expressed cre in parvalbumin interneurons.

(B) 5ms pulses of blue (473nm) light elicited strong hyperpolarizing postsynaptic currents at in pyramidal cells at -50 mV. Holding the cell at the reversal potential for chloride completely abolished postsynaptic currents.

(C) Gabazine, a GABAa receptor blocker, blocked light induced responses indicating that they are GABAergic in nature.

(D) mPFC neurons transfected with ChR2 under the CamKIIa promotor. Pyramidal neurons showed

strong depolarizing currents upon pulses of blue light.
(F) Current clamp recordings revealed a minor depolarizing membrane potential.
(G) Reliability of ChR2 induced spiking in mPFC pyramidal neurons.
(H) Low reliability of spiking suggets a prominent role for inhibition elicited by surrounding pyramidal neurons. Indeed, depolarizing the cell to -50 mV revealed hyperpolarizing currents
pyramidal cells that fired unreliably.
(I) Blocking GABAa receptors with gabazine increased reliability of firing.

interneurons is less clear. In the cortex regular spiking cells receive more interlaminar excitation compared to intralaminar excitation (Xu and Callaway, 2009) and have a lower connectivity probability with pyramidal neurons (Otsuka and Kawaguchi, 2009). Also from hippocampal studies we know that these cells show a much lower connectivity with other cells in the network and receive less asymmetrical inputs compared to parvalbumin interneurons. CCK cells respond less readily to excitatory input. Recruitment of glutamatergic inputs onto CCK cells is highly depressing indicating that they cannot follow high frequency inputs. These properties make them fire less reliable in relation to fast network oscillations (Freund and Buzsaki, 1996; Bartos and Elgueta, 2012). RSNP neurons might be important in integrating signals over longer time periods. Another interesting possibility would be that they are mainly subjected to recruitment by neuromodulators and could thereby serve to modify circuits during different behavioral state. RSNP or NFS cells are strongly recruited by acetylcholine through β2 receptors and could potentially serve an important role in regulating network dynamics during attention behavior.

Specificity in regulating pyramidal neuron output by β2 containing interneurons might arise from (i) the specificity in pyramidal neurons populations they target or (ii) by restricted activation of basal forebrain inputs of certain cell types in the PFC. The first hypothesis is obviously easier to test. To better define the role of β2 receptors in regulating prefrontal cortical activity it might be in particular interesting to investigate connectivity to defined pyramidal neuron populations. By fluorescent retrograde labeling of prefrontal projection neurons one can define if β2 interneurons preferentially target pyramidal neurons depending on the area to which they send their information (Kassam et al., 2008). Since we found that across cortical layers NFS cells consist of two kinds of populations, defined by the presence or absence of the β2* receptor, it will be exciting to see if these are embedded in different functional networks. The projection neurons to the ventral striatum would serve a nice entry point for investigating functional connectivity between β2-containing interneurons and defined pyramidal neurons as discussed above. These experiments could add to the question whether inhibition is a global phenomenon or shows a stereotypic connectivity depending on output of the circuit. Somatostatin neurons show high divergent connectivity with pyramidal neurons and in addition are interconnected through gap junctions, therefore they might serve a global role in preventing over excitation and suppression of further dendritic inputs. RSNP cells might show lower

connectivity rate and therefore are recruited specifically when acetylcholine levels rise during attention. Hypothetically they might serve a more refined role in favoring one output neuron over the other depending on the connectivity. Our data points to a general role of β2* receptors to alter the balance of excitation to inhibition during attentional tasks which might affects integration of inputs to the network and generate appropriate output of the circuit. Obviously, the rich source of new cre-driver lines will allow for assessment of genetically identified neurons and their role in attention (Taniguchi et al., 2011). This will allow for direct testing of the hypothesis that inhibition plays a role in attention. An attempt in that direction has been made. For example, parvalbumin interneurons can be specifically targeted and their involvement in attention behavior can be assessed (Figure 3). Since task associated activity is seen in preparation before cue presentation and after cue presentation it allows for the first time to rapidly and reversibly assess the contribution of these specific cell types of the PFC in these different phases of the behavioral task. With optogenetics prefrontal cortical elements can be controlled rapidly, reversible and with millisecond precision. A similar configuration can be used for the assessment of inputs coming into the PFC and the role of acetylcholine release. This will allow for testing the hypothesis that inhibitory tone in the PFC regulates goal-directed behavior and when altered leads to shifts in behavior. Needless to say that optogenetics will start to yield exciting new hypotheses and data that is easier to interpret compared to lesion and pharmacological studies performed so far. This will constitute an important step in defining the construct and circuitry of attention.

Role of inhibition in information processing in the PFC

An important way in which GABAergic interneurons could affect afferent input processing in dendrites of PFC neurons is by affecting spike-timing dependent plasticity. Synaptic plasticity is critically important for cognitive function, and in particular, synaptic plasticity in the PFC has been directly associated with attention and working memory (Laroche et al., 2000). The relative timing of action potentials in pre- and postsynaptic neurons has a profound impact on the induction of long-term potentiation or depression. When a presynaptic spike precedes a postsynaptic spike within a short time window of several tens of milliseconds, LTP is induced. The reverse order of spike-timing results in long term depression (LTD) (Markram et al., 1997; Bi and Poo, 1998). In mouse PFC, nicotine strongly affects this timing-dependent synaptic plasticity, which is called spiketiming- dependent plasticity (STDP). Stimulation of nicotinic AChRs in PFC modifies STDP induced by pairing stimulation of the excitatory inputs to PFC layer 5 pyramidal neurons with postsynaptic spikes elicited 5 ms after each synaptic response (Chapter 4). This coordinated stimulation induced robust LTP; however, when the same stimulus paradigm was applied in the presence of nicotine concentrations experienced by smokers, LTP was eliminated and

a depression of the excitatory inputs to these cells was observed. Which nAChRs on what neurons are responsible for this effect? As discussed above, mouse PFC layer 5 pyramidal neurons do not express nicotinic receptors themselves. Rather, the nAChRs involved in the nicotinic modulation of LTP induction increase inhibitory GABAergic inputs to the pyramidal cells, as the nicotinic modulation of plasticity was abolished by inhibitors of GABA type A (GABAa) receptors. As described above, LTS and RSNP GABAergic interneurons found in the PFC layer 5 express nAChR subunits on their soma that activate these neurons when nicotine is present. FS interneurons are excited indirectly by nAChRs that increase glutamatergic excitation of those cells. Thus, nicotine exposure enhances inhibitory input to the layer V pyramidal neurons through both direct and indirect excitation of inhibitory GABA interneurons. Studies in other cortical areas indicate that increases in postsynaptic calcium concentration are critical for the induction of synaptic plasticity (Koester and Sakmann, 1998; Sjostrom and Nelson, 2002). Using two-photon imaging of intracellular calcium levels, it was found that action potentials that propagated from the soma into the dendrites of layer 5 pyramidal cells elicited increases in dendritic calcium concentration. Nicotine enhanced the GABA input to the same dendrites, resulting in less calcium entry, likely due to failure of action potential back-propagation from the soma. Thus, nicotine suppresses postsynaptic calcium changes, thereby altering the conditions necessary for synaptic potentiation. Burst-like stimulation of the pyramidal cell in the presence of nicotine could restore postsynaptic calcium to concentrations comparable to those seen in the absence of nicotine, as well as the STDP, indicating that strong postsynaptic stimulation could overcome the nicotinic modulation (Couey et al., 2007).

The activation of distributed nAChRs provides the PFC neuronal network with a wide range of computational possibilities, but the functional consequences of this modulation are hard to predict from these data alone. Nicotine alters the rules for synaptic plasticity resulting from timed presynaptic and postsynaptic activity and increases LTP threshold by reducing dendritic calcium signals. As such, the function of the medial PFC network will most likely change in the presence of nicotine. Presumably, distal apical dendrite of layer 5 pyramidal neurons in superficial layers will be more quantitatively affected by the nicotinic mechanisms we found to block STDP than the synapses that are located closer to the cell body. By reducing dendritic action potential propagation in apical dendrites, nicotine hampers communication between cell body and distal synapses in layer 5 pyramidal neurons. This potentially could strongly affect information processing in the neuronal network of the medial PFC as a whole, and will alter the output of the PFC. At the same time, increased activity in pyramidal neurons restores the conditions for STDP to occur. The presence of nicotine and increased threshold for STDP could reduce cognitive performance in healthy naive rodents (Day et al., 2007). Alternatively, since PFC neuronal activity could be increased during PFC-based cognitive behavior, nicotine may provide conditions under which signal-to-noise ratio in PFC information processing is

enhanced, thereby improving cognitive performance (Mirza and Stolerman, 1998; Day et al., 2007). It is possible that enhancing signal-to-noise for phasic activity within the PFC, rather than simply increasing excitability, could be an effective mechanism for cognition-enhancing drugs.

Similar mechanisms most likely act in concert in other layers of the PFC that alter synaptic learning rules after nicotinic receptor stimulation. Positive timing intervals also potentiate synapses in layer II-III of the mPFC (Meredith et al., 2007). In addition, similar inhibitory mechanisms act in layer II-III of the cortex to inhibit dendrites of pyramidal neurons (Kapfer et al., 2007). Hence most likely in superficial layers the threshold for inducing plasticity is also increased. nAChRs placed on other compartments might also contribute to plasticity. Interestingly we found that Layer V pyramidal neurons also express α7 receptors postsynaptically (Poorthuis et al., 2013b). This nicotinic receptor is highly permeable for calcium and is therefore able to potentiate glutamatergic synapses (McGehee et al., 1995; Ji et al., 2001). Hence depending on the location of cholinergic stimulation, i.e. the postsynaptic dendrite or the presynaptic interneuron, acetylcholine release might have opposing effect on spike-timing dependent plasticity. This hypothesis can be tested using mice expressing channelrhodopsin under de ChAT promoter and locally release acetylcholine around dendrites or connected interneurons. To disentangle the role of these opposing processes it will be interesting to investigate the location on the dendrite that receives increased inhibition and the distribution of α7 receptors on the dendrite in relation to distinct excitatory inputs coming into the prefrontal cortex.

Nicotine desensitizes nicotinic receptors in the PFC

While nicotine on the short-term can alter synaptic transmission and plasticity rules, cigarette smoking leads to a prolonged presence of nicotine levels in the brain that reach 300 nM for minutes (Matta et al., 2007). Smoking of one cigarette leads to nearly complete β2* nAChR receptor saturation in humans (Brody et al., 2006). Sustained exposure to low levels of nicotinic agonists rapidly desensitizes nicotinic receptors (Fenster et al., 1997; Picciotto et al., 2008). Whether nicotine concentrations seen by smokers desensitize nAChRs in circuits involved in attention behavior was not known. We find that nicotine rapidly decreases responsiveness of β2* nAChRs in the PFC, while leaving α7 nAChRs intact. Because of co-application of ACh and nicotine we cannot rule out agonist competition at the receptor binding site, however the persistent reduced responsiveness of β2* nAChRs (over 45 minutes) after the presence of nicotine suggests that nicotinic receptors indeed were desensitized. An alternative explanation could be that nicotinic receptors were internalized (John and Gordon, 2001). However, the responses did recover after an hour, suggesting recovery from desensitization. The subunit specificity of receptor desensitization observed is similar to that seen in the ventral tegmental area where nicotine desensitizes β2*

nAChRs on GABAergic interneurons, but not α7 nAChRs on glutamatergic terminals and dopamine neurons (Mansvelder et al., 2002; Wooltorton et al., 2003). Hence, whereas α7 nAChRs display rapid desensitization kinetics after being activated by rapid increases in agonists, they do not desensitize upon the prolonged presence of smoking concentrations of nicotinic agonist. These separate processes, referred to as 'classical' and 'high-affinity' desensitization (Giniatullin et al., 2005), thus operate in the PFC as well suggesting that α7 nAChRs remain available for activation by fast cholinergic transients during smoking concentrations of nicotine (Parikh et al., 2007).

The desensitizing properties of β2* nAChRs are heterogeneous. The accessory α5 subunit plays a critical role in determining whether β2* nAChRs remain available for cholinergic signaling (Bailey et al., 2010; Grady et al., 2012). In the cortex α5 subunits are preferentially expressed by neurons in deep layers (Winzer-Serhan and Leslie, 2005). Expression of α5 subunits is lower in superficial layers (Winzer-Serhan and Leslie, 2005), but still α5 could be located on NFS interneurons, which constitute a small number of cells in the PFC modulated by β2* nAChRs (Poorthuis et al., 2012). In the PFC, α5 and β2 subunits co-assemble in layer VI pyramidal neurons (Bailey et al., 2010). We find that the presence of α5 subunits does not extend to NFS interneurons in layer VI, which show a higher and complete degree of desensitization after nicotine exposure. However, it has been reported that some cortical interneurons express β2 and α4 subunits in combination with α5 subunits (Porter et al., 1999). We find that β2-mediated responses in LV interneurons show similar levels of desensitization as responses by LVI pyramidal neurons, suggesting that they may also express α5 subunits.

At the network level desensitization of nicotinic receptors give clear prediction about altered functioning of cellular processing. For example, cholinergic control over inhibitory circuits is absent in superficial layers and diminished in layer V. Hence, acetylcholine cannot increase inhibitory tone to increase signal-to-noise ratio in these layers after smoking concentrations of nicotine. It renders the system incapable of regulating dendritic inhibition and subsequently might alter spike-timing dependent plasticity mechanisms. In addition acetylcholine is less able to stimulate thalamic terminals to increase the weight of these synapses integrated in the PFC circuitry.

That desensitization happens in vivo seems obvious, but how this might contribute to behavior remains elusive. β2* Receptor activation is strongly compromised and absence of this receptor leads to a strong impairment in attention (Guillem et al., 2011), at first hand it would be logic to assume that nicotine worsens attention performance. However, effects of nicotine on behavior are rather variable and can improve attention, worsen attention or have no effect (Hahn et al., 2003; Levin et al., 2006; Pattij et al., 2007; Bailey et al., 2010). It is hard to make a clear prediction on what the mechanisms of nicotine are in modulating attention, predominantly due to the widespread direct cellular effects on nicotinic receptors, but also their role in influencing release of other neuromodulators that can have a long-lasting effects and

may explain behavioral effects of nicotine (Hahn and Stolerman, 2005).

Functional adaptations to nicotine exposure and relevance for attention behavior.

Nicotine concentrations found in peripheral bloodstream during smoking a single cigarette strongly desensitize nicotinic receptors in the prefrontal cortex (Poorthuis et al. 2013). This raises the question how the nicotinergic system adapts to the presence of nicotine to compensate for this loss in cholinergic signaling. In contrast to other systems which reduce their receptor levels in response to overexposure to agonist, nicotinic receptor agonists increase the number of receptors (Wonnacott, 1990). This suggests that desensitization in response to prolonged presence of nicotine, and hence a reduction in functional cholinergic signaling, triggers a process that increases receptor levels. Supporting this hypothesis, it has been shown that concentrations of nicotine that induce desensitization are equally effective in inducing receptor upregulation (Fenster et al., 1999) and antagonists induce upregulation in similar amount and comparable brain regions (Pauly et al., 1996). Nicotine concentrations reached during smoking a single cigarette saturate β2* nAChRs in the human brain (Brody et al., 2006). Nicotine concentrations build up during the day to reach sustained levels 60-300 nM (Matta et al., 2007). This suggests a tight interplay between desensitization and subsequent upregulation of receptor levels in smokers. In post mortem cortical tissue of human subjects that smoked, increased levels of nicotinic receptor binding sites were found and the amount of upregulation correlates with smoking history (Benwell et al., 1988; Breese et al., 1997). Imaging studies showed that receptors remain upregulated even in the abstinence of smoking for more than seven days (Staley et al., 2006). Upregulation after chronic nicotine exposure has also been shown in rodents (Flores et al., 1992; Picciotto et al., 2008).

Subtypes of nAChRs have a different affinity for nicotine (McGehee and Role, 1995) and desensitization properties of nAChRs are subunit dependent (Fenster et al., 1997; Mansvelder and McGehee, 2002). This suggests that upregulation of nAChRs is also subunit dependent. Our results show that in the PFC network low affinity α7 nAChRs activation is not hampered in the continuous presence of nicotine, whereas high-affinity β2* nAChRs show strong degrees of desensitization. In addition, the degree β2* nAChR desensitization depends on the presence of the α5 subunit. This is in agreement with consistent findings that high afiinity β2 nAChRs are upregulated in smokers and laboratory animals exposed to nicotine (Paterson and Nordberg, 2000; Picciotto et al., 2008). How does this relate to the prefrontal cortex network? It has been shown that exposure to nicotine also increases nicotinic receptor binding in the prefrontal cortex. The increased binding was paralleled with increased levels of β2 and α4 subunits, but not α7 subunits. In addition, α5 subunits were not upregulated (Counotte et al., 2012b). Hence this parallels the finding that α5 subunits protect β2* nAChRs from desensitization and α7 nAChRs are not desensitized by chronic

exposure to nicotine (Bailey et al., 2010; Poorthuis et al., 2013a). nAChRs subtypes that require higher levels of nicotine to desensitize (α3β4) also require higher doses of nicotine to upregulate this receptor subtype, again arguing that desensitization triggers upregulation (Fenster et al., 1999).

Upregulation of α4β2 nicotinic receptors is found in the absence of an increase in mRNA (Pauly et al., 1996). Several other posttranslational mechanisms have therefore been proposed to underlie increased nAChR binding sites (Govind et al., 2009). Exposure to nicotine might induce conformational changes in the receptor that could decreases receptor turnover (Peng et al., 1994) or stabilizes nicotinic receptors in a high-affinity state (Vallejo et al., 2005). In addition, nicotine might enter the cell to enhance maturation of receptors in the endoplasmatic reticulum (Sallette et al., 2005) where it can act as a chaperone to enhance receptor assembly (Kuryatov et al., 2005; Lester et al., 2009). An increase number of cell surface receptors might depend on exocytotic mechanisms (Harkness and Millar, 2002). A question that is remaining is whether the increased nicotinic binding sites yield an increase in functional receptors and what the consequence thereof is for cognitive functioning. Nicotine exposure indeed leads to increased nAChR functioning in the PFC. β2* nAChRs in LII-III enhance GABAergic transmission to pyramidal neurons and after nicotine exposure the augmentation of GABAergic transmission is enhanced (Counotte et al., 2012b). This is in line with our findings that superficial layers in the PFC most strongly desensitize by exposure to nicotine (Poorthuis et al. 2013). It remains to be determined whether cells that express α5 subunits show upregulation. However, α5 containing receptors are relatively resistant to desensitization (Kassam et al., 2008) and have been suggested to be resistant in upregulation in vivo (Mao et al., 2008). As outlined above, in our studies we found higher levels of desensitization with somatic puffs compared to bath application of acetylcholine in other studies (Kassam et al., 2008) and the relatively little desensitization in the imaging experiments. This may suggest that there is a differential distribution of α5 containing nAChRs along the somatodendritic axis, with more α5 containing receptors located on dendritic compartments. These receptors might be differentially sensitive to upregulation. At the behavioral level increased receptor number in the PFC might contribute to reduced attention performance during withdrawal from nicotine (Shoaib and Bizarro, 2005; Semenova et al., 2007; Picciotto et al., 2008).

What are the consequences of repeated nicotine exposure for circuit development and attention behavior in the long-term? During repeated exposure to nicotine receptors undergo cycles of activation and desensitization. Since β2 receptors on GABAergic neurons most strongly desensitize compared to pyramidal neurons in layer VI, nicotine might lead overall to an overexcited PFC. This might be counterbalanced by an increase in mGluR levels seen right after nicotine exposure (Counotte et al., 2011). When time progresses mGluR levels decrease which might be in response to a network that receives higher levels of inhibition due to upregulation of receptor

on interneurons (Counotte et al., 2012b). Hence desensitization might trigger a cascade of events involving mGluR upregulation, nicotinic receptor upregulation and subsequent mGluR downregulation. The persistent downregulation of mGluR levels have been shown to cause impaired attention performance during adulthood (Counotte et al., 2011). In addition, synaptic learning rules are altered due to altered mGluR function which might underlie the decrements in attention (Goriounova and Mansvelder, 2012b). In conclusion, nicotine exposure during adolescence leads to a lasting decrease in attention performance which might be caused by altered information processing in the mPFC (Counotte et al., 2012a; Goriounova and Mansvelder, 2012a).

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Nicotinic acetylcholine receptor modulation of attention behavior and prefrontal cortical circuits

At every moment in time, our brain receives numerous sensory information about the environment. This makes attention, the process by which we select currently relevant stimuli for processing and ignore irrelevant input, a fundamentally important brain function. By sustaining your attention you can structure your behavior in time to attain a fututre goal. The prefrontal cortex ia a central structure in the brain involved in regulating attention. In humans we test attention in the 'Continous performance task'. This task has also been adapted for rodents, the 5-choice serial reaction time task, and allows us to study the molecular and cellular mechanisms underlying attention. In these tasks humans and rodents need to report, over a longer period of time, the appearance of rare stimuli. By studying how well they do this, we can assess attention. This thesis tries to contribute to the understanding of the cellular mechanisms through which nicotinic acetylcholine receptors modulate attention.

Acetylcholine is a neurotransmitter that translates a chemical signal in an electrical signal and thereby changes the computational properties of neurons. There is a lot of evidence for the rol of acetylcholine in attention. The dynamics of acetylcholine differ per brain area. During attention there is a special dynamics in the prefrontal cortex and many experiments show that acetylcholine exerts its main effect here. The fast dynamics of cholinergic signals suggest that the nicotininc acetylcholine receptor, a fast ionotropic receptor, could play an important role.

This thesis answers a couple of questions regarding the function of nicotinic receptors in attention and the regulation of the underlying neuronal networks. Firstly, there are several types of nicotinic receptors, are these all important? We show that nicotinic receptors containing 'β2 subunits' play a main role in orchestrating attention. In addtion, we show that the neurons to which this receptor transmits its signal are located in the prelimbic area of the prefrontal cortex.

To understand how this nicotinic receptor influences cortical computation we looked at where these receptors are located in the network. The cortex is build up of different layers. Al these layers have their own specific connections with other brain areas. This thesis answers the question whether these layers are differentially regulated by nicotinic receptors. The answer is yes and the main reason is that pyramidal neurons, neurons that send glutamatergic signals to other brain areas, are differentially regulated by nicotinic receptors in the different layers. In superfical layers they are not regulated by nicotinic receptors, midle layer pyramidal neurons contain α7 receptors and deep layers contain β2 receptors. In addition, activity of pyramidal neurons is fine-tuned by interneurons. We found that interneurons throughout all layers are regulated by nicotinic receptors. Hence they play an important role in regulating

neuronal activity in the prefrontal cortex.

Nicotinic receptors are not only senstive to endogenous chemicals from the body but also for substances that appear elsewhere in nature, like nicotine. Nicotine is a psychoactive substance that activates our reward centre in the brain en that makes it addictive. Nicotine also has an effect on cognitive functions like attention. In certain patient populations nicotine can improve attention. Networks of cells can change the strength of connections between neurons. This makes it possible to assign new functions to networks, for example to remember new things. This so called plasticity is also important during attention. If an input is integrated by a neuron and followed by neuronal output (an action potential), synapses can change strength. We describe that nicotine influences this process by increasing inhibition in the prefrontal cortex. As a consequence, calcium signals in the dendrite are lower and synapses can not become potentiated. This could be a mechanism through which the prefrontal cortex increases its signal-to-noise ratio and consequently not every input leads to a change in synaptic strength in the network.

If the brain is exposed to nicotine over longer periods, in particular during adolescence, this can lead to a decrement in attention performance. A couple of adaptations have been described that change the prefrontal cortical network. These include an increase in the number of nicotinic receptors and a change in metabotropic glutamate receptors. The initial mechanisms that lead to these adaptations remained unknown. In this thesis I show that nicotine strongly interferes with cholinergic signaling in the PFC. It does this mainly by making β2 receptors less sensitive for acetylcholine. This effect is not everywhere the same and some cell types suffer more from this then others. Mainly interneurons show diminshed cholinergic responsiveness. This finding leads to the hypothesis that during nicotine exposure the prefrontal cortex is hyperexcited. This could in turn lead to a compensatory mechanisms increasing nicotinic receptors and increasing the levels of metabotropic glutamate receptors.

These findings contribute to the question how acetylcholine orchestrates attention behavior and prefrontal cortical circuitries. In addition, they show how nicotine can alters these circuits on the short- and long-term. A better understanding of the cellular mechanisms of nicotinic receptor modulation of attention behavior can ultimately lead to better targeted treatment of attention disorders and in particular Alzheimer's disease, in which the cholinergic system is malfunctioning.

De rol van nicotine acetylcholine receptoren in de modulatie van attentie gedrag en neuronale circuits in de prefrontale cortex

Attentie maakt het mogelijk om ons te focussen op omgevingsstimuli die op dit moment belangrijk zijn en irrelevante informatie te onderdrukken. Door 'je aandacht erbij te houden' kan je langere tijd je gedrag structureren om een doel in de toekomst te bereiken. De prefrontale cortex is een centrale structuur in het brein die het langer vast houden van aandacht mogelijk maakt. Bij mensen kunnen we aandacht testen in de 'continous performance task'. Van deze taak is ook een versie voor knaagdieren, de '5-choice serial reaction time task', die het mogelijk maakt moleculaire en cellulaire mechanismes te bestuderen die te grondslag liggen aan aandacht. In deze taken moeten mensen of knaagdieren over langere tijd de aanwezigheid van een bepaalde stimulus rapporteren. Door te kijken hoe goed ze dit doen kunnen we aandacht bestuderen. Dit proefschrift tracht bij te dragen aan het begrip van de cellulaire mechanismes waarmee nicotine acetylcholine receptoren aandacht regelen.

Acetylcholine is een neurotransmitter die een chemisch signaal omzet in een elektrisch signaal en daarmee de rekeneigenschappen van neuronen veranderd. Er is veel bewijs voor de rol van acetylcholine en het reguleren van attentie. De dynamiek van acetylcholine afgifte verschilt per hersengebied. Tijdens attentie is er een speciale dynamiek in de prefrontale cortex en veel proeven laten zien dat acetylcholine hier een groot deel van zijn effect op attentie uitvoert. Aangezien het acetylcholine signaal een snelle dynamiek heeft zouden nicotine receptoren weleens een belangrijke rol kunnen spelen in het vertalen van dit signaal naar veranderde neuronale activiteit die ten grondslag ligt aan attentiegedrag. Maar hoe dit gebeurt is tot op heden niet bestudeerd.

Dit proefschrift beantwoordt een aantal vragen omtrent de functie van nicotine receptoren in attentiegedrag en het reguleren van de onderliggende neuronale netwerken. Ten eerste: er zijn een aantal verschillende nicotine receptoren, zijn deze allemaal even belangrijk voor attentie? We laten zien dat nicotine receptoren die opgebouwd zijn met behulp van 'β2 subunits' voornamelijk een prominente rol spelen in het reguleren van attentie. Tevens laten we zien dat de neuronen waaraan deze receptor zijn signaal doorgeeft tijdens attentie gesitueerd zijn in het prelimbisch gebied van de prefrontale cortex.

Om te begrijpen hoe deze nicotine receptor het neuronale netwerk beïnvloedt, hebben we daarna gekeken waar deze neuronen gesitueerd zijn. De hersenschors is opgebouwd uit verschillende lagen. Al deze lagen hebben hun eigen specifieke verbindingen met andere hersengebieden. Dit proefschrift beantwoordt de vraag of de verschillende lagen anders gereguleerd worden door nicotine receptoren. Het antwoord hierop is ja en ligt hem vooral in het feit dat pyramidaal cellen, glutamaterge neuronen die voornamelijk informatie naar andere hersengebieden zenden, in de verschillende lagen anders gereguleerd worden. In ondiepe lagen worden pyramidaal

cellen niet gereguleerd door nicotine receptoren., in de middenlaag door α7 receptoren en in diepe lagen door β2 receptoren. Daarnaast wordt de activiteit van pyramidaal cellen op het juiste niveau afgesteld door interneuronen. We hebben gevonden dat interneuronen door alle lagen heen gereguleerd worden door nicotine receptoren en ze dus een belangrijke rol spelen in het reguleren van activiteit in de prefrontale cortex.

Nicotine receptoren zijn niet alleen gevoelig voor lichaamseigen stoffen als acetylcholine, maar worden ook geactiveerd door stoffen die elders in de natuur voorkomen, zoals nicotine. Nicotine is een stof die ons beloningscentrum activeert en daardoor verslavend is. Nicotine heeft echter ook een effect op cognitieve functies zoals attentie. In bepaalde patiëntenpopulaties kan nicotine zelfs attentie verbeteren. Netwerken kunnen de sterkte van verbindingen aanpassen. Dit maakt het mogelijk om netwerken een nieuwe functie te geven, bijvoorbeeld voor het onthouden van nieuwe gebeurtenissen. Plasticiteit is waarschijnlijk ook belangrijk tijdens attentie. Als een signaal geintegreerd wordt door een neuron en daarbij direct gevolgd door neuronale output (een actie potentiaal), dan kunnen synapsen zich versterken. We beschrijven dat nicotine gevolgen heeft voor het induceren van deze vorm van plasticiteit door inhibitie in de prefrontale cortex te verhogen. Dit heeft tot gevolg dat calcium signalen in de dendriet lager zijn en synapsen zich niet meer versterken. Het zou een mechanisme kunnen zijn waarmee de signaal/verhouding in de prefrontale cortex omhoog gaat en dus niet iedere input zomaar tot een verandering in het netwerk leidt.

Als het brein echter over langere periodes blootgesteld wordt aan nicotine, en dan met name tijdens ontwikkelingsperiodes als de adolescentie, kan dit tot vermindering van attentie leiden. Er zijn een aantal adaptaties op de lange termijn beschreven die het prefrontale cortex netwerk doen veranderen, zoals een vermeerdering van receptoren en verandering in het metabotrope glutamaat receptor eiwit. De initiële mechanismes die leiden tot deze adaptaties bleven echter onbekend. In dit proefschrift laten we zien dat nicotine sterk interfereert met cholinerge signalen in de prefrontale cortex, vooral door β2 receptoren minder gevoelig te maken voor acetylcholine. Dit effect is niet overal even groot en sommige cel types hebben een sterkere vermindering dan anderen. Vooral interneuronen hebben een sterke vermindering, meer dan pyramidaal cellen. Deze vinding leidt tot de theorie dat gedurende nicotine blootstelling de prefrontale cortex hyperactief wordt en dat zou kunnen leiden tot een verhoging van nicotine receptoren op interneuronen en het vermeerderen van het aantal metabotrope glutamaat receptoren. Dit zou de eerste stap kunnen zijn in de cascade van pathofysiologische gevolgen van blootstelling aan nicotine tijdens de adolescentie.

Deze vindingen dragen bij aan de vraag hoe acetylcholine attentie reguleert door middel van nicotine receptoren. Tevens laat het zien hoe nicotine het netwerk op korte en lange termijn kan veranderen. Een beter begrip van de cellulaire mechanismes waardoor nicotine receptoren attentie en neuronale circuits in de prefrontale cortex reguleren kan bijdragen aan gerichtere behandelingen van attentiestoornissen die te maken hebben met het acetylcholine systeem, zoals bijvoorbeeld Alzheimer's.

To my father and mother: -This is where the many Sunday trips have come together. You gave me the chance to explore the world. Although I think we might be different in many things, it probably only takes time to realize I am not. Thank you.

Jacintha, my friend and lover. Your rational mind sets my intuitive mind at ease. I always thought you had more of a 'science-brain' then me, and secretely you contributed to the projects in this thesis. I am forever thankful for the way you supported me in whoever I was, thought to be or will become. Your patience tolerated my many moods and what I call my 'mobile-identity'. You are on my side for so long, no doubt we are gonna make it.

Take care and so long,

Rogier, Zandvoort 2013